Controlled Cultivation Techniques for the
Recovery of Threatened Fishes in Virginia

by

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CONTROLLED CULTIVATION TECHNIQUES FOR THE RECOVERY OF THREATENED FISHES IN VIRGINIA

by

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(ABSTRACT)

The goal of this research was to develop captive propagation methods for the Federally threatened spotfin chub, Cyprinella monacha, and yellowfin madtom, Noturus flavipinnis, by using closely related, but unthreatened species, to evaluate potential techniques. The surrogate species used were the whitetail shiner, Cyprinella galactura, and the margined madtom, Noturus insignis. I investigated methods to promote gonadal maturation, induce spawning, and rear larvae of these fishes.

Captive whitetail shiners developed mature gonads under a variety of temperature and photoperiod conditions. Spawning condition was maintained for over two years when they were held at constant warm temperature (≈ 24 C) and long photoperiod (16 h light). Whitetail shiners did not readily spawn in aquaria, but were induced to spawn by hormonal injection with human chorionic gonadotropin (hCG) and carp pituitary extract (CPE) at mean dosages of 1688 I.U./kg and 20 mg/kg, respectively, or with luteinizing hormone releasing hormone analogue (LHRHa) and domperidone at mean dosages of 363 μg/kg and 36 mg/kg, respectively. Most females spawned within 30 h of the first injection. Stripped ova were effectively wet-spawned, and larvae hatched in 8 d at 25 C. I obtained a mean hatch rate of 55 %, but lack

of swimbladder inflation resulted in very poor survival of several batches of eggs. Larvae began feeding within 2 d of hatching, and survival rates of 50 to 90 % after 16 d were obtained when larvae were fed twice daily on a diet of brine shrimp nauplii at a rate of ≈ 10/L/d, and a commercially prepared larval fish diet at a rate of ≈ 14 mg/L/d.

Changing photoperiod, but not temperature, was required to induce oocyte maturation in most captive female margined madtoms. Sperm production in mature male madtoms was enigmatic; motile sperm were observed only once. Plasma testosterone concentrations in males peaked just prior to the spawning season at 6.5 ng/mL, but levels were not correlated with male gonadosomatic values. Plasma 17β-estradiol levels in females peaked just prior to the spawning season at 15 ng/mL, and were correlated with gonadosomatic values. Captive margined madtoms did not tank spawn unless they were hormonally injected with hCG and CPE at mean dosages of 5256 I.U./kg and 58 mg/kg, respectively, or with LHRHa and domperidone at mean dosages of 554 μg/kg and 55 mg/kg, respectively. Most females ovulated within 78 h of the first injection. Inclusion of more than one breeding pair per tank inhibited tank spawning. Embryos did not develop in 55 % of tank-spawned ova, or from any strip-spawned ova. Parents consumed spawned egg masses if they remained with the nest. Hatch rates > 65 % were obtained by suspending egg masses in a large-mesh basket over turbulent aeration at 28 to 30 C. Larvae hatched in 7 d at 28 C. Survival rates > 50 % after 15 d were obtained when larvae were fed salmon starter twice daily at a rate of 20 mg/L/d, and tanks were thoroughly cleaned daily.

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INTRODUCTION

Three species of Virginia fishes, the spotfin chub, <u>Cyprinella</u> <u>monacha</u>, slender chub, <u>Erimystax</u> <u>cahni</u>, and yellowfin madtom, <u>Noturus</u> <u>flavipinnis</u>, are Federally listed as threatened. Captive propagation is listed as a strategy for preservation in the Federal recovery plans for all three of the threatened fishes. Successful propagation can facilitate recovery of these rare fishes to non-threatened status, if reared fishes are stocked into historic habitats that have been restored for naturally reproducing populations. Stuart and Johnson (1981) state that the ultimate goal of captive breeding of rare fishes is to return them to suitable habitats in the wild, and that it provides excellent opportunities for research into species biology.

A negative aspect of captive breeding of rare fishes is that it requires removal of fish from depauperate wild populations for spawning, without assurance that progeny will be produced in sufficient numbers to strengthen source populations or establish new populations. Recent population surveys of the three threatened fishes of Virginia indicate that they are too rare to be used experimentally for developing captive breeding techniques. The slender chub is the rarest of the three fishes; none have been observed in Virginia since 1986. Only two yellowfin madtoms have been reported in Virginia since 1987. I have recently observed the spotfin chub in the North Fork Holston, River, Virginia, but it is not common. Consequently, common congeneric species were used as surrogates to develop protocols for propagation of

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these rare fishes. The whitetail shiner, Cyprinella galactura, was selected as the surrogate species for the spotfin chub; the streamline chub, Erimystax dissimilis, and blotched chub, Erimystax insignis, were selected as surrogates for the slender chub, and the margined madtom, Noturus insignis, was selected as the surrogate for the yellowfin madtom. Dr. Robert Jenkins (Roanoke College, Salem, Virginia), an ichthyologist and expert on Virginia fishes, was consulted in the selection of surrogate species to help ensure that their spawning requirements and behaviors were similar to those of the target species.

Efforts to rear the streamline chub and blotched chub were abandoned relatively early; these species are very sensitive to electrofishing (mortality of collected fish typically > 50 %) and are difficult to maintain for long periods of time in the laboratory. Thus, attempts to captively propagate the slender chub should not be undertaken until these problems and other problems are worked out by the use of surrogate species.

Protocols developed to propagate the spotfin chub and yellowfin madtom have good potential to facilitate propagation of other rare fishes. Half of the 24 described species of Cyprinella are rare enough to have legal protection or to be of special concern (Johnson 1987, Robins et al. 1991, Table I.1). Six species of Cyprinella have been spawned in captivity with apparent ease, but the methods to spawn adults and rear larvae have not been critically examined (Hubbs and Strawn 1956, Pflieger 1965, Gale and Gale 1977, Gale and Buynak 1978, Gale 1986, Heins and Rabito 1986,

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Table I.1. Status of North American cyprinids in the genus <u>Cyprinella</u>. List of all <u>Cyprinella</u> spp. obtained from Robbins et al. (1987), and threatened status determined from Johnson et al. (1990).

Common	Rare locally	Rare Federally
<u>Cyprinella</u> <u>callisema</u>	<u>Cyprinella</u> <u>analostana</u>	<u>Cyprinella</u> <u>caerulea</u>
<u>Cyprinella</u> <u>chloristia</u>	<u>Cyprinella</u> <u>callistia</u>	<u>Cyprinella</u> <u>callitaenia</u>
<u>Cyprinella</u> <u>galactura</u>	<u>Cyprinella</u> <u>camura</u>	<u>Cyprinella</u> <u>formosa</u>
<u>Cyprinella</u> <u>gibbsi</u>	<u>Cyprinella</u> <u>lutrensis</u>	<u>Cyprinella</u> <u>monacha</u>
<u>Cyprinella</u> <u>labrosa</u>	<u>Cyprinella</u> <u>spiloptera</u>	<u>Cyprinella</u> <u>proserpina</u>
<u>Cyprinella</u> <u>leedsi</u>	<u>Cyprinella</u> <u>venusta</u>	
<u>Cyprinella</u> <u>lepida</u>	<u>Cyprinella</u> <u>whipplei</u>	
<u>Cyprinella</u> <u>nivea</u>		
<u>Cyprinella</u> <u>pyrrhomelas</u>		
<u>Cyprinella</u> <u>trichroistia</u>		
<u>Cyprinella</u> <u>xaenura</u>		
<u>Cyprinella</u> <u>zanema</u>		

Heins 1990). It is logical to conclude that a synthesis of information on propagation of captively bred <u>Cyprinella</u> spp., in conjunction with the fragmentary information on reproductive ecology of <u>Cyprinella</u> spp., can help identify promising propagation techniques for rare species.

Twenty-five of the 40 species of Ictaluridae are rare enough to have legal protection or to be of special concern (Johnson 1987; Robins et al. 1991). The rare catfishes include two species of <u>Ameiurus</u>, two species of <u>Ictalurus</u>, 18 species of <u>Noturus</u>, and the monotypic genera <u>Pylodictis</u>, <u>Satan</u>, and <u>Trogloglanis</u> (Table I.2).

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Table I.2. Status of North American freshwater catfishes, Ictaluridae. Species list of ictalurids from Robbins et al. (1987), and status of species determined from Johnson et al. (1990).

Common	Rare locally	Rare Federally
Ameiurus brunneus	Ameiurus natalis	Ictalurus pricei
Ameiurus catus	Ameiurus nebulosus	Noturus baileyi
Ameiurus melas	Ictalurus furcatus	Noturus flavipinnis
Ameiurus platycephalus	Noturus eleutherus	Noturus furiosus
Ameiurus serracanthus	Noturus exilis	Noturus gilberti
Ictalurus lupus	Noturus flavus	Noturus lachneri
Ictalurus punctatus	Noturus hildebrandi	Noturus munitus
Noturus albater	Noturus miurus	Noturus placidus
Noturus elegans	Noturus phaeus	Noturus stanauli
Noturus flavater	Noturus stigmosus	Noturus taylori
Noturus funebris	Noturus sp.	Noturus trautmani
Noturus gyrinus	Pylodictis olivaris	Satan eurystomus
Noturus insignis		Trogloglanis pattersoni
Noturus leptacanthus		
Noturus nocturnus		

Aside from several species of Ameiurus, Ictalurus, and Pylodictis, little is known of factors that regulate reproduction of ictalurids, and few attempts at controlled propagation of noncommercial catfishes have been made. Thus, protocols to artificially propagate rare ictalurids are unavailable. A critical review of the large volume of literature on propagation of commercially important ictalurids, in

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conjunction with information on natural history and controlled reproduction of other ictalurids, would help to identify promising propagation techniques for rare ictalurids.

The goal of this research was, therefore, to develop viable protocols to propagate rare species of Cyprinella and Noturus. This dissertation is divided into chapters that address three topics critical to the development of protocols to propagate rare species of Cyprinella and Noturus; maturation of gonads, spawning, and rearing of larvae and fry. Detailed observations and results of my research experiments with the whitetail shiner and the margined madtom are included in all chapters. Chapters 1, 2, and 3 focus on Cyprinella spp., and Chapters 4, 5, and 6 are devoted to the madtoms.

Methods to induce and maintain spawning condition of captive brood stock are described in Chapters 1 and 4. Most experiments explored the effects of temperature and photoperiod on recrudescence of the gonads. Chapter 1 includes a discussion of several interactions which were investigated between males and females relative to development of spawning condition, and in Chapter 4, changes in levels of steroids in the blood relative to state of gonadal maturation, and the effect of prolonged hormonal therapy on gonadal development are described.

Chapters 2 and 5 focus on the development of spawning techniques for captive fish. The potential for natural spawning in captivity, and the potential of hormonal injections to induce spawning were evaluated. Original observations and an

assessment of methods for hatching eggs for each group of fishes are included in these chapters.

Finally, methods to rear larvae to an advanced fry stage are examined in Chapters 3 and 6. The potential use of dry diets or brine shrimp as foods for larvae and fry of Cyprinella spp. and Noturus spp. was explored, and the systems used to maintain early life stages of the whitetail shiner and margined madtom are described and evaluated.

CHAPTER 1

Control of gonadal maturation of <u>Cyprinella</u> <u>galactura</u>

<u>Introduction</u>

Control of gonadal maturation is requisite to controlled spawning of captive fishes. The goal of this research was to develop a method to induce and maintain spawning condition in captive <u>Cyprinella</u> spp. by using the whitetail shiner, <u>Cyprinella</u> <u>galactura,</u> as a model species. Several investigations (objectives) were completed to achieve this goal. They are listed below along with very brief rationale and purpose statements. Several exploratory experiments are included because the information they provide aided in the development of recommendations to induce and maintain maturation of <u>Cyprinella</u> spp.

The exploratory experiments were initiated primarily to address observations and subsequent questions that arose as other experiments progressed. Also included is a description and analysis of gonadal characteristics deemed relevant to assess state of gonadal maturation, and facilitate spawning of <u>Cyprinella</u> spp.

<u>Temperature-Photoperiod Experiments</u>

The two primary factors controlling maturation of fish gonads in temperate regions are photoperiod and temperature (de Vlaming 1972, Crim 1982). Out-of-

season maturation of captive fish, and subsequent out-of-season spawning, can be achieved through manipulation of these environmental cues (Harrington 1950, Horvath 1986). Several Cyprinella spp. have been spawned out-of-season (Pflieger 1965, Gale 1986). This objective assessed the effects of several combinations of modified temperature and photoperiod cycles on maturation of the gonads of whitetail shiners.

Description of Gonads and Gametes

The gonads and gametes of female and male whitetail shiners have not been described in detail. This objective compared the morphology of the gonads and gametes of the whitetail shiner with those of other teleosts.

Correlation Between Degree of Tuberculation and GSI Values of Males

The existence of a good relationship between external breeding characteristics, such as tuberculation, and the gonadosomatic index (GSI) values of male Cyprinella spp. could aid in the selection of males for induced spawning trials. This objective determined the strength of the relationship between these two morphological variables.

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Correlations Between Oocyte Diameter and GSI Values of Females

Useful information on spawning condition, and subsequent selection of fish for hormonal induction of spawning, can be obtained by examination of samples of oocytes. Size of oocytes and the position of the germinal vesicle within the oocytes are particularly useful indicators of readiness to spawn (Conte et al. 1988, Rottmann and Shireman 1988). This objective evaluated relationships between oocyte diameter, germinal vesicle position, and spawning success of hormonally-injected whitetail shiners.

Effects of Isolating Females from Males

In preliminary holding trials, feeding of female whitetail shiners seemed to be inhibited by males. This objective determined whether condition factor and spawning condition of females isolated from males was superior to that of nonisolated females.

Maturation of Fish Captured in Mid-winter

Several fish captured during mid-winter for other experiments were introduced into a tank with an ongoing temperature and photoperiod experiment. The purpose of this objective was to determine the ability of these fish to achieve spawning condition.

Maturation of Captive Fish at Constant Temperature and Photoperiod

Adult red shiners, _Cyprinella_ _lutrensis_, can be maintained in spawning condition for years under a regime of constant long photoperiod and warm temperature (Gale 1986). This study determined the ability of out-of-season fish to achieve spawning condition under continued exposure to constant temperature and photoperiod.

Development of New Complements of Mature Ova in Females

Cyprinella spp. females spawn several clutches of eggs during the spawning season (Rabito and Heins 1985, Gale 1986), but reports indicate that the number of days required to develop a new clutch of mature oocytes, and subsequent frequency of spawning, varies among different species (Gale and Gale 1977, Gale and Buynak 1978, Heins and Rabito 1986). This objective estimated the time required for whitetail shiner females to develop a new complement of mature oocytes following hormonal injections.

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Materials and Methods

Fish used in all experiments were collected from sandy shorelines along the New River or from a pool on the lower end of Spruce Run, a small tributary to the New River in Montgomery County, Virginia. At high water, whitetail shiners from the river enter the pool and become trapped when the water level recedes. Because no whitetail shiners were collected above the pool, all fish used in experiments were considered to be of New River origin. Fish were collected by seining (9 m X 2 m seine with 8 mm bar mesh) at night and were prophylactically treated for disease after capture and periodically thereafter. Treatments consisted of baths of salt (0.2 %) and Furacin (5 to 10 mg/L) for at least 4 h, and formalin for at least 4 h (25 mg/L) or for 1 h (250 mg/L).

Captive fish were fed flake food (Zeigler Brothers Prime Flake Food) ad libitum once or twice daily. Feedings of flake food were frequently supplemented by a 3 mm diameter moist pellet. The moist pellet was prepared by grinding whole, fresh or frozen fish with commercial trout or catfish feed in a commercial meat grinder equipped with a 3 mm extruder plate.

In several experiments, the criterion used to judge the effects of treatments was whether or not the fish were in 'spawning condition'. Unless stated otherwise, males were considered to be in spawning condition when they were brightly colored and had pronounced cephalic tubercles. Females were considered to be in spawning condition when they had distended abdomens and pronounced ovipositors.

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Hereafter, males and females judged to be in spawning condition are referred to as spawners, and those judged not to be in spawning condition are referred to as nonspawners.

Tanks used in experiments included 280 L (1.5 X 0.5 X 0.5 m) and 530 L (2.1 X 0.6 X 0.6 m) rectangular fiberglass tanks (Living Streams), a screened-off section of an 8000 L (10.0 X 1.0 X 0.8 m) rectangular concrete raceway, and screened-off sections of a 7000 L (17.0 m circumference X 1.2 m width X 0.4 m deep) oval fiberglass raceway.

Temperature-Photoperiod Experiments

Photoperiod was controlled by several timers and incandescent lights, or by a computer-controlled dimmer and incandescent lights. In both systems, the lighting intensity was gradually changed over a period of at least 0.5 h hour at the beginning and end of each daylight period. The gradual change was deemed necessary to preclude startling and subsequent stressing of fish used in experiments. Photoperiod for each day was calculated by adding one hour of light (i.e., a 0.5 h dawn and 0.5 h dusk period) to lengths of days (sunrise to sunset) recorded in The Farmer's Almanac. The times were corrected for latitude.

Water temperature was controlled by Frigid Unit water chillers, inputs of well water, adjustment of room temperature in environmental chambers, or with a large refrigeration unit attached to the oval raceway. The environmental chambers were

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2.4 m X 2.4 m insulated rooms in which air temperature was thermostatically regulated by air-conditioning and heating units. Heated water was not required because the warmest temperatures did not exceed ambient room temperatures.

Temperature and photoperiod were manipulated in four ways: 1) natural cycles were simulated; 2) natural cycles were compressed from time of capture to a time when expected natural spawning conditions were reached; 3) natural cycles were compressed, but began with mid-winter conditions and then continued to a time when expected natural spawning conditions were reached; and 4) temperature and photoperiod were held constant at levels characteristic of the natural spawning season for 175 d. Natural spawning conditions were considered to be 15 to 16 h light and 22 to 23 C. The ability of whitetail shiners to develop mature gonads under controlled conditions was evaluated in a series of experiments that used eight combinations of the temperature and photoperiod regimes previously described. The combinations of temperature and photoperiod were as follows:

1) WF - Wild fish under a natural photoperiod and temperature regime (Figure 1.1). The first collection of wild fish was in October.

2) NT-NP - Captive fish held under simulated natural conditions (Figure 1.2). Fish were collected and confined in October.

3) UET-NP - Captive fish held under uniform elevated temperature (19 to 24 C) and natural photoperiod. Fish were collected and confined in June.

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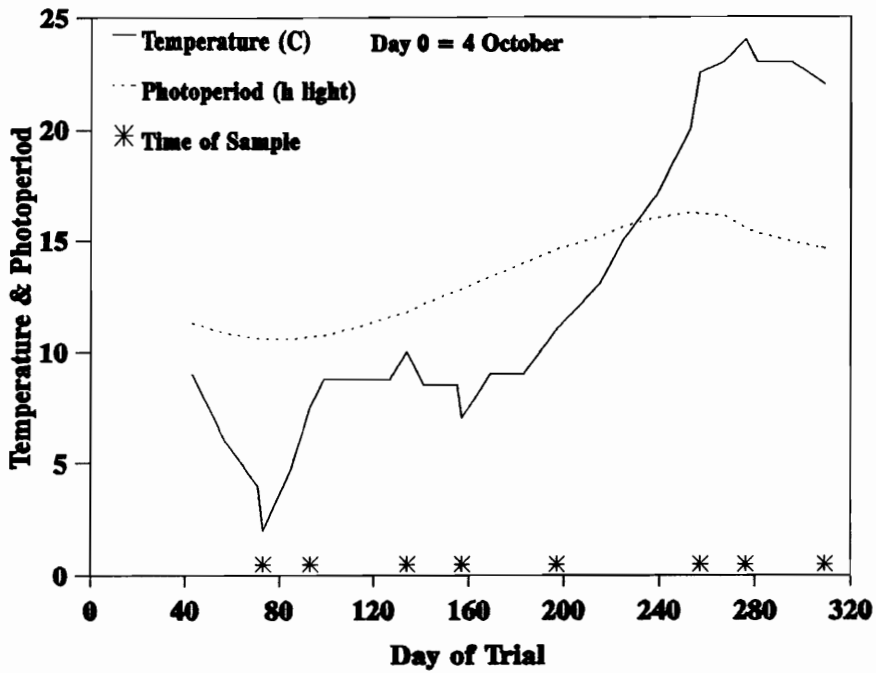
Figure 1.1. Approximate temperature and photoperiod regimes for fish collected from the wild.
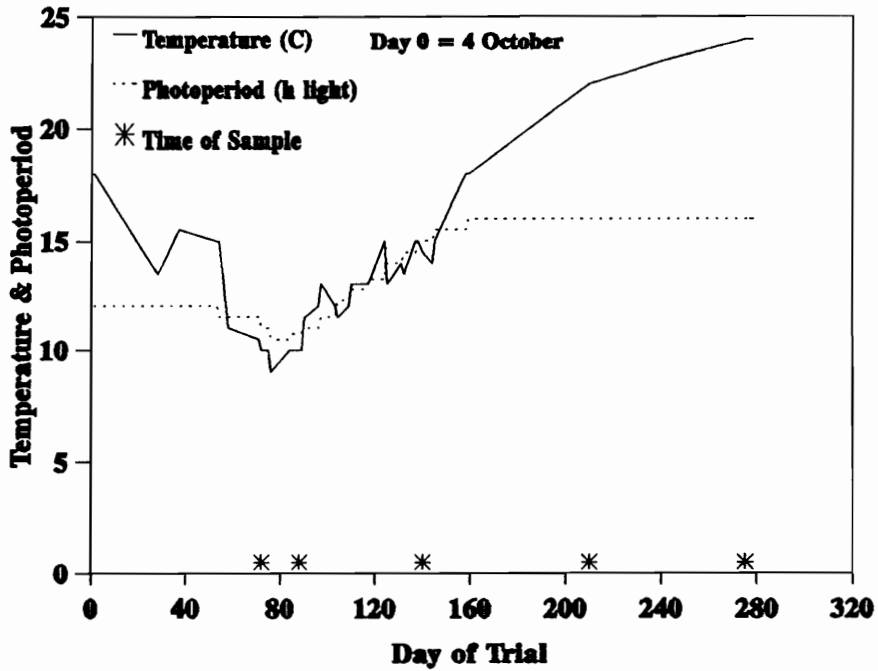
Figure 1.2. Approximate temperature and photoperiod regimes for captive fish held under simulated natural temperature and photoperiod (Experiment NT-NP).

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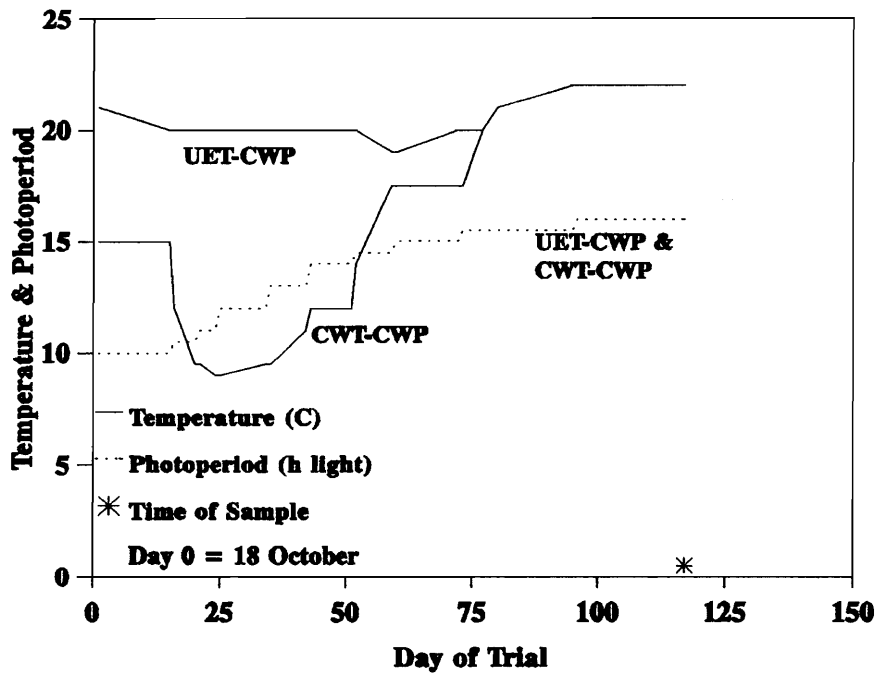
Figure 1.3. Approximate temperature and photoperiod regimes for captive fish held under a compressed photoperiod cycle, and exposed to either a compressed temperature cycle or constant temperature (Experiments CWT-CWP and UET-CWP).

4) UET-CWP - Captive fish held under uniform elevated temperature and a compressed photoperiod cycle that started with a mid-winter photoperiod cycle (Figure 1.3). Fish were collected and confined in October.

5) CWT-CWP - Captive fish held under compressed temperature and photoperiod cycles that started with a mid-winter temperature and photoperiod cycle (Figure 1.3). Fish were collected and confined in October.

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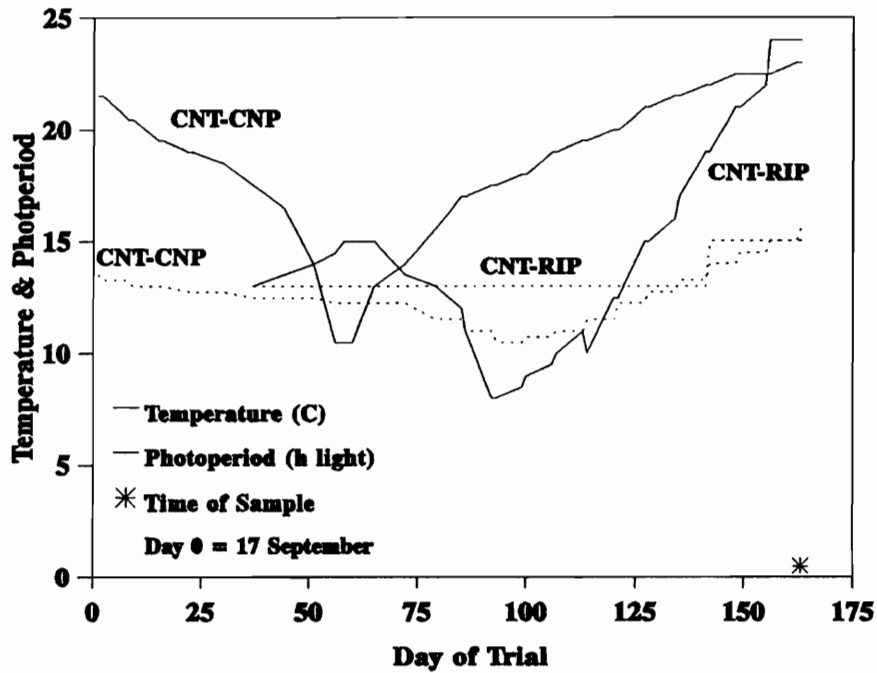
Figure 1.4. Approximate temperature and photoperiod regimes for captive fish held under a compressed temperature cycle, and exposed to either a rapid increase in photoperiod or a compressed photoperiod cycle (Experiments CNT-CNP and CNT-RIP).

6) UET-ULP - Captive fish held under uniform elevated temperature (19 to 23 C) and a uniform long (16 h light) photoperiod. Fish were collected and confined in August.

7) CNT-CNP - Captive fish held under compressed natural temperature and photoperiod cycles (Figure 1.4). Fish were collected and confined in September.

8) CNT-RIP - Captive fish held under a compressed natural temperature cycle and exposed to a rapid increase in photoperiod (Figure 1.4). Fish

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Table 1.1. Summary of experiments to evaluate the effects of photoperiod and temperature on the maturation of <u>Cyprinella galactura</u> gonads.

Experiment	Temperature[1]	Photoperiod[1]	Number of days	Beginning (ending) date	Tank size (L)[1]	Number of fish[2]
WF	Natural	Natural	270	14 Nov (10 Aug)	NA[3]	NA
NT-NP	Simulated natural	Simulated natural	275	4 Oct (5 Jul)	7000	109
UET-NP	Uniform elevated	Simulated natural	122	15 Jun (14 Oct)	8000	30
UET-CWP	Uniform elevated	Compressed winter	117	18 Oct (11 Feb)	530	69
CWT-CWP	Compressed winter	Compressed winter	117	18 Oct (11 Feb)	530	102
UET-ULP	Uniform elevated	Uniform long (16 h)	175	15 Aug (5 Feb)	7000	44
CNT-CNP	Compressed natural	Compressed natural	148	17 Sep (11 Feb)	530	103
CNT-RIP	Compressed natural	Rapid increase	110	25 Nov (11 Feb)	350	24

[1]See text for more detailed descriptions of temperature-photoperiod regimes and tank types
[2]Number of fish stocked at beginning of each experiment
[3]Not applicable

were collected and confined in November.

The number of fish used in each experiment depended on the number that could be captured at the time of the beginning of the experiment, and ranged from 24 to 109 (Table 1.1). Differences in gonadosomatic indices (GSI = (ovary weight/ body weight (including viscera) $\times$ 100) of males and females were used to assess the effectiveness of each temperature and photoperiod combination to induce gonadal

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maturation. Eight GSI samples of fish in Experiment WF and four samples in Experiment NT-NP were collected at regular intervals. GSI samples for all other experiments were collected only at the end of the experiments. Because fish used in experiments were not collected at exactly the same time or from the same exact locations, it is possible that the morphological or physiological state of the fish varied among groups at the beginning of the experiments. Consequently, GSI values among experiments were not compared statistically with a general linear model (GLM). Instead, plots were constructed to visually assess differences among treatments, and the results of each treatment (only the July sample from Experiment NT-NP) were compared statistically to that of the July sample of wild fish (Experiment WF) using Student's t-test, to determine whether gonadal development of the captive fish was comparable to that of wild fish captured during the spawning season.

Description of Gonads and Gametes

Ovaries and testes of whitetail shiners preserved in 10 % formalin were exposed by removing a portion of the abdominal wall, and the position and attachments of the gonads to other parts of the body were then determined. Motility of sperm of males used in spawning experiments was periodically checked by viewing samples of milt or a macerated section of a testis under a light microscope (100X to 250X), and then adding fresh water to activate the sperm. Morphology of spermatozoa was determined by examination under a light microscope (1000X) and

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reviewing photographs (400X). The head diameters and tail lengths of whitetail shiner spermatozoa were determined by measurements obtained from the photographs. Motility of sperm in fresh water and phosphate buffered saline was compared. Additional information on the gonads and gametes, relative to GSI values, is contained in the next two sections.

Correlation Between Degree of Tuberculation and GSI Values of Males

The intensity of tuberculation and coloration, relative to GSI values of males in several of the temperature-photoperiod experiments, was qualitatively assessed. Each fish was assigned a score of one to four: one was assigned to fish with no tuberculation and nonbreeding coloration; two, to fish with light tuberculation and little or no breeding coloration; three, to moderately tuberculated fish with obvious breeding coloration; and four, to highly tuberculate, brightly colored fish. The values for intensity of tuberculation and coloration were plotted with corresponding GSI values for each fish. Lines were then fit by eye to delineate the approximate GSI values that correlated with spawning condition in whitetail shiner males. Percent of males in spawning condition in each of the temperature-photoperiod experiments was then estimated using this value.

Coloration of nonbreeding whitetail shiner males is silver with a light blue sheen. In breeding males, the head and fins take on a salmon-pink coloration, and the fins have a pronounced white edge. Intensity of tuberculation of highly

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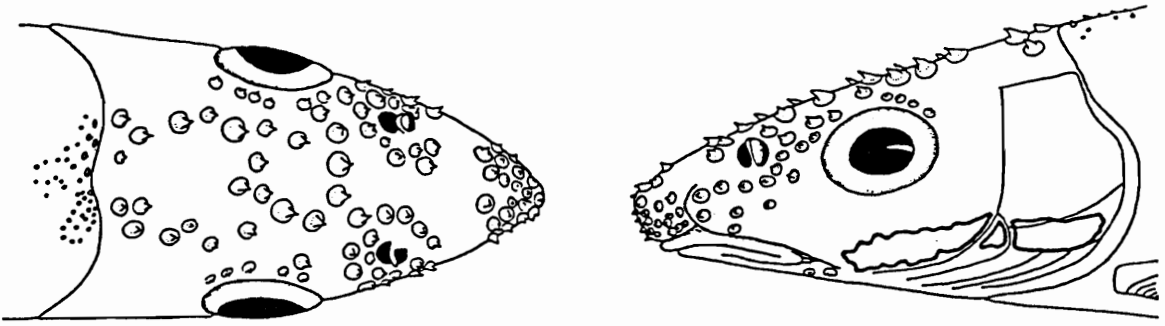
Figure 1.5. Illustration of a highly tuberculate male spotfin chub, Cyprinella monacha, (From Jenkins and Burkhead 1984).

tuberculate male whitetail shiners is similar to that of the spotfin chub, Cyprinella monacha, as illustrated by Jenkins and Burkhead (1984, Figure 1.5).

Correlations Between Oocyte Diameters and GSI Values of Females

The number of size classes of vitellogenic oocytes of females from seven of the eight temperature-photoperiod experiments was determined by a gross examination of the ovaries under a binocular microscope at 20X. The diameters of the largest size class of oocytes were measured with the aid of an ocular micrometer. Mean diameters of largest size class of oocytes were not compared statistically among treatments with a GLM, because fish were collected at different times and locations, and it is possible that the morphological or physiological state of the fish varied among groups of fish at the beginning of the experiments. However, the mean value from each experiment was evaluated to determine whether the oocytes were

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approaching the minimum size that will respond to hormonal injections ($\approx$ 1 mm, see Chapter 2).

The ovaries of a subsample of 20 fish that had been preserved in 10 % formalin were examined to determine the effects of position within the ovary on mean oocyte size. To ensure representation of all stages of development, ovaries with oocytes at disparate stages of maturation (i.e., sizes) were intentionally selected for the analysis. Four biopsy samples of oocytes were taken equidistant along the length of each ovary with a small cork borer. Diameters of thirty oocytes from each biopsy were then measured, and the mean diameter of the oocytes from each biopsy was plotted for all fish. A GLM, blocked for effects of individual fish, was used to test whether differences in mean oocyte diameters among biopsy positions were statistically significant.

Size-frequency distributions of diameters of oocytes obtained from the biopsy samples (N = 120 for each ovary) were plotted to illustrate changes that occur as oocytes mature within the ovaries of whitetail shiners. The plots were arranged from smallest to largest mean oocyte diameter, to help demonstrate development of additional size classes of oocytes within the more mature ovaries. The ordering of samples is not necessarily contingent upon time or experiment. Size-frequency distributions of samples with the four smallest mean oocyte diameters were not plotted, but the distributions were very similar to those of other samples that contained relatively small oocytes (i.e., females #5, #6, and #7).

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The number of oocytes of different sizes per weight of ovary also was determined for the 20 fish from which ovarian biopsy samples had been taken. Small pieces of ovary from each fish were removed with a scalpel. Each piece was weighed, and the number of oocytes in each was counted. In ovaries that contained relatively large oocytes, a noticeable break in the size distribution of oocytes occurred at a diameter of $\approx$ 0.50 mm, and consequently, oocytes > 0.50 mm in diameter could be readily distinguished from smaller classes of oocytes. It was very time consuming to count oocytes < 0.50 mm in all samples, because this size class was so numerous. Therefore, to facilitate processing of samples, only oocytes > 0.50 mm were counted in more developed ovaries (N = 11); otherwise, all oocytes > 0.10 mm were counted (N = 9).

The weight of oocytes < 0.50 mm in more developed ovaries was considered negligible when compared to that of the larger oocytes and ovarian tissues. In female common carp, Cyprinus carpio, in spawning condition, 40 to 70 % of the ovarian mass consists of fully yolked oocytes, and the remainder is mostly ovarian tissue (Horvath 1986). Regressions of the natural log (ln) of the number of oocytes per milligram of ovarian tissue versus egg diameter were then constructed from the data. The regression equations were used to estimate fecundity (number of oocytes > 0.1 mm or number of oocytes > 0.5 mm) of 97 whitetail shiner females for which mean diameters of the largest size class of oocytes had been determined by measurement of 8 oocytes. The natural log of the number of oocytes per milligram

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of ovarian tissue was used, because increase in weight of oocyte per unit of increase in length was expected to change in a logarithmic fashion. Estimated fecundities for fish with mean oocyte diameters > 0.50 mm were regressed against weight of females to develop an equation for fecundity based on body weight of female whitetail shiners. The weight-fecundity equation was transformed to a length-fecundity equation by converting the weight variable to length with a length-weight regression developed from 36 gravid females (i.e., GSI values > 4.0).

Effects of Isolating Females from Males

Fifty-two female whitetail shiners that had been held with males in the 7000 L oval raceway, at a constant temperature (22 C) and photoperiod (16 h light) for several months, were isolated from the males by a screened divider. Spawning condition of females and males was evaluated prior to separation of the sexes. The two groups were isolated for 65 d, and then spawning condition of all fish was reevaluated. After reexamination, half of the females were kept in isolation and the others returned to the section containing males. Spawning condition of fish was reevaluated again after 75 d. Condition factors of females were determined at this time. Mean condition factors of the two groups were compared statistically using Student's t-test. Temperature was 22 C, and photoperiod was 16 h light during both holding periods.

Maturation of Fish Captured in Mid-Winter

Forty-five fish were captured and placed in a section of the 7000 L oval raceway after the initiation of Experiment NT-NP. Thirty-six of these fish were captured on day 40 (November) of Experiment NT-NP, and the remaining nine were captured on day 133 (February) of Experiment NT-NP. These fish were exposed to the same temperature and photoperiod regime as fish in Experiment NT-NP (Figure 1.2). Spawning conditions in the tank (16 h light and 22 C) were reached on day 210. Beginning on day 250, spawning condition of the fish was examined periodically. Spawners were removed and used in experiments (see Chapter 2) to test the efficacy of hormonal injections to induce spawning of captive whitetail shiners.

Maturation of Captive Fish at Constant Temperature and Photoperiod

Spawning condition of 118 fish that had been held for several weeks or months at a constant temperature (22 C) and photoperiod (16 h light) in the 7000 L oval raceway was assessed at the end of the natural spawning season. Of those examined, 30 males and 33 females were spawners (53% of all fish). Spawners were separated from nonspawners, and the spawning condition of individuals in each group of fish was assessed 54, 96, and 175 d after the separation. All fish were retained at a constant temperature (22 C) and photoperiod (16 h light) throughout the holding period.

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Development of New Complements of Mature Ova in Females

Fish used in spawning experiments typically were released into a section of the 7000 L oval raceway at the conclusion of experiments. After hormonal injections, there are few if any viable mature oocytes because they typically are spawned or undergo atresia. Oocytes from females used in three spawning experiments were examined to determine whether the females had developed a new complement of ova. The females had been injected with either luteinizing hormone releasing hormone analogue plus domperidone or human chorionic gonadotropin plus carp pituitary extract 7 to 25 d prior to the examination (see Chapter 2 for explanation of injection procedure and dosages). Additionally, three females and three males used in another spawning experiment were isolated in an aquarium at the conclusion of the experiment; larger oocytes from all of the females were atretic at that time. After 27 d, oocyte samples were taken to determine whether the females had developed a new complement of mature oocytes.

Results

Temperature-Photoperiod Experiments

The GSI values of males and females collected from the wild (Figures 1.6 and 1.7) were similar to those of males and females in Experiment NT-NP (Figures 1.8 and 1.9) at comparable time periods. The mean GSI values for wild fish were highest in the 5 July sample (day 234). GSI values for Experiments WF and NT-NP were notably variable for samples collected during spawning conditions. GSI values of wild males tended to vary more than those of NT-NP males, whereas the opposite was true for females. GSI values for females in the other six experiments tended to be higher in fish exposed to a uniform elevated temperature (Figure 1.10). Males did not exhibit this tendency (Figure 1.11). Notable within sample variability of GSI values among males and females occurred in all experiments except UET-NP.

Only the mean GSI value of males from Experiment CWT-CWP differed statistically from that of wild fish ($p = 0.002$). The high variability of GSI values within treatments likely was the primary factor that additional statistically significant differences were not observed (Table 1.2). It is worth noting that the two lowest mean GSI values were from Experiments CWT-CWP and UET-CWP (Table 1.2). In addition to having identical photoperiod, fish in these experiments were relatively crowded; initially 102 and 69 fish per tank, respectively. Fish in Experiment CNT-CNP were also fairly crowded, with 103 fish at the beginning of the experiment.
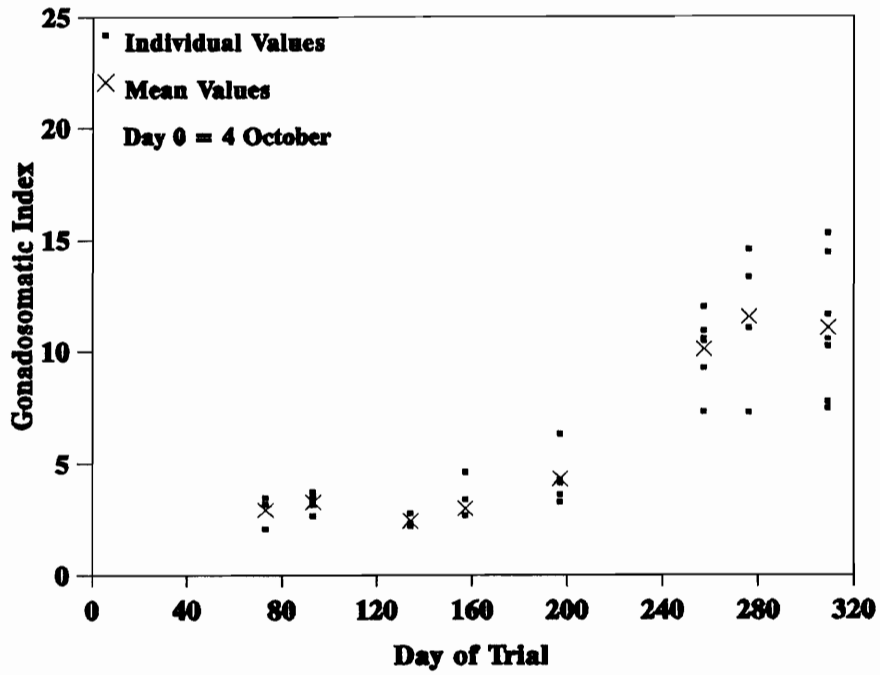
Figure 1.6. Gonadosomatic indices of female <u>Cyprinella</u> <u>galactura</u> collected from the wild.
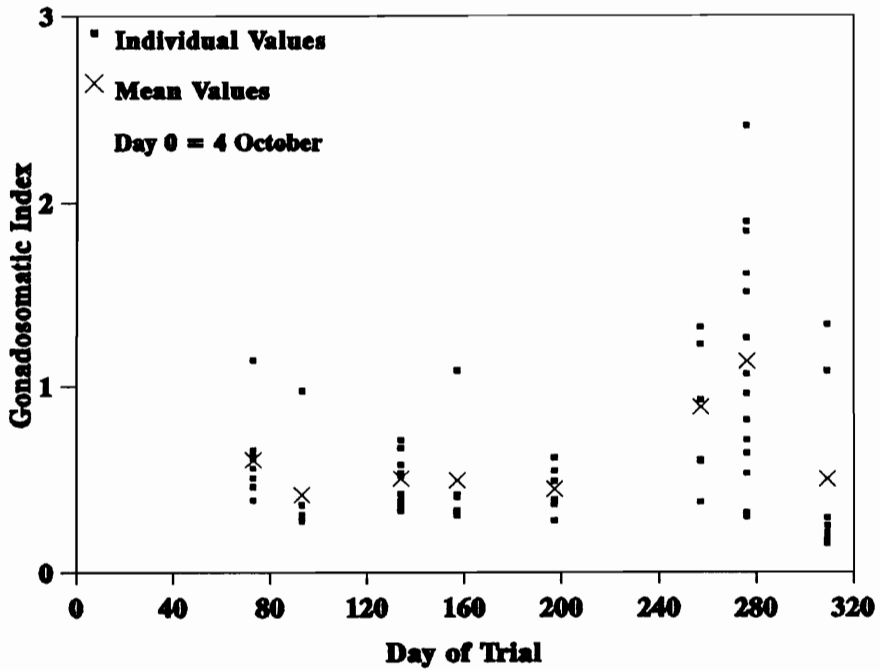
Figure 1.7. Gonadosomatic indices of male <u>Cyprinella</u> <u>galactura</u> collected from the wild.

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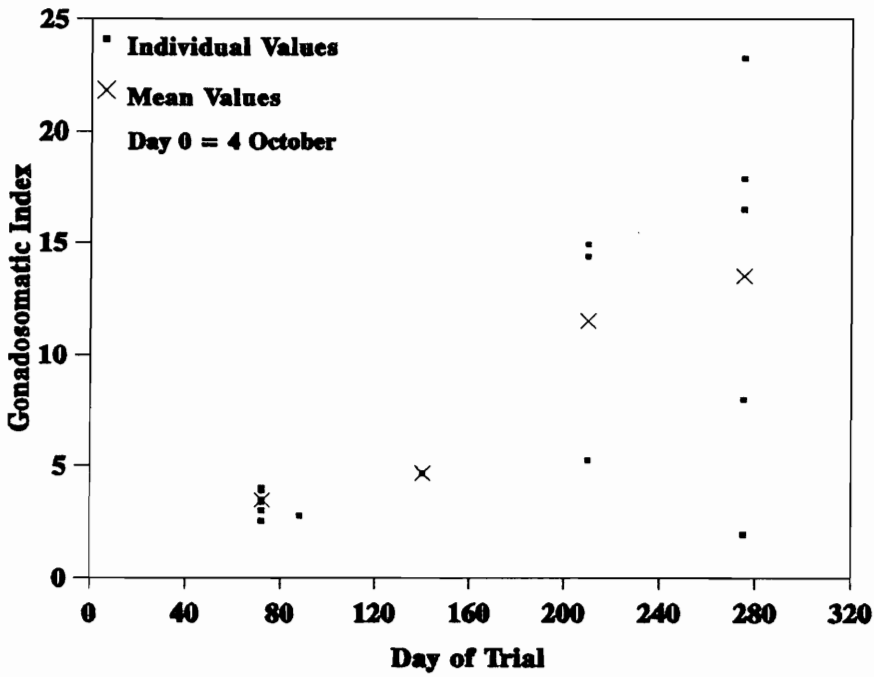
Figure 1.8. Gonadosomatic indices of captive female <u>Cyprinella</u> <u>galactura</u> held under a simulated natural temperature and photoperiod regimes (Experiment NT-NP).
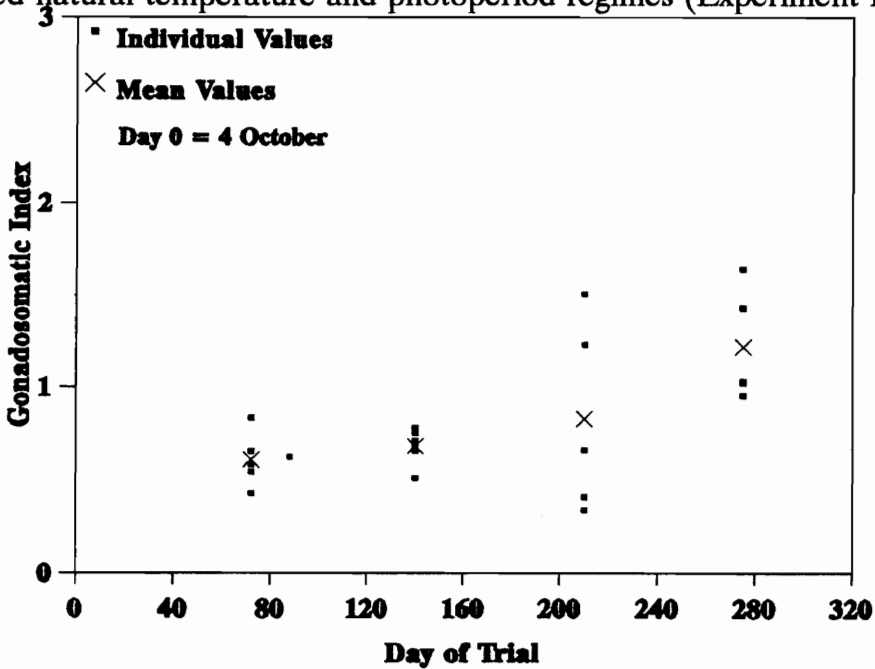
Figure 1.9. Gonadosomatic indices of captive male <u>Cyprinella</u> <u>galactura</u> held under simulated natural temperature and photoperiod regimes (Experiment NT-NP).

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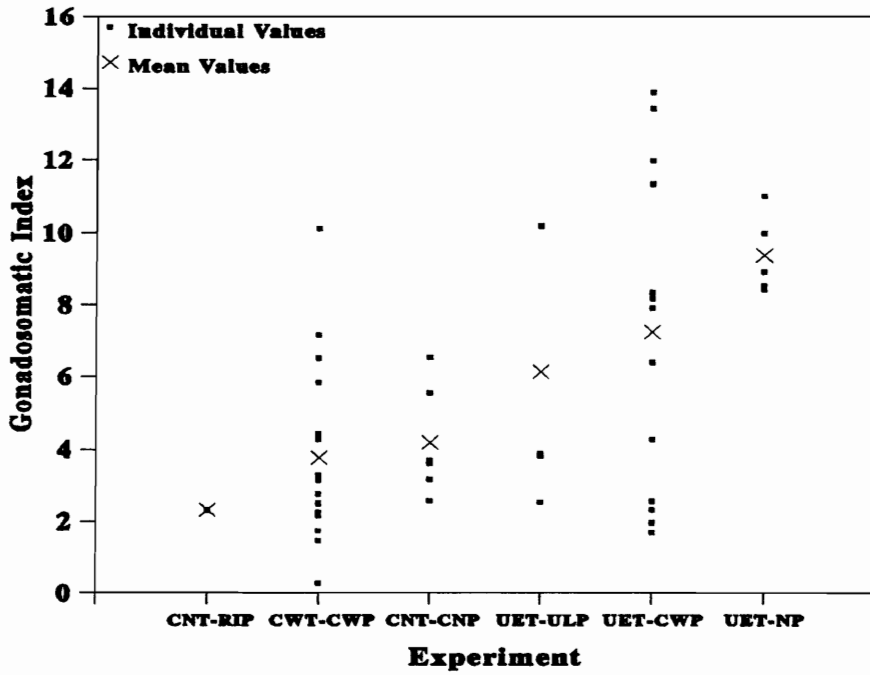
Figure 1.10. Gonadosomatic indices of captive female <u>Cyprinella</u> <u>galactura</u> held under six different temperature and photoperiod regimes.
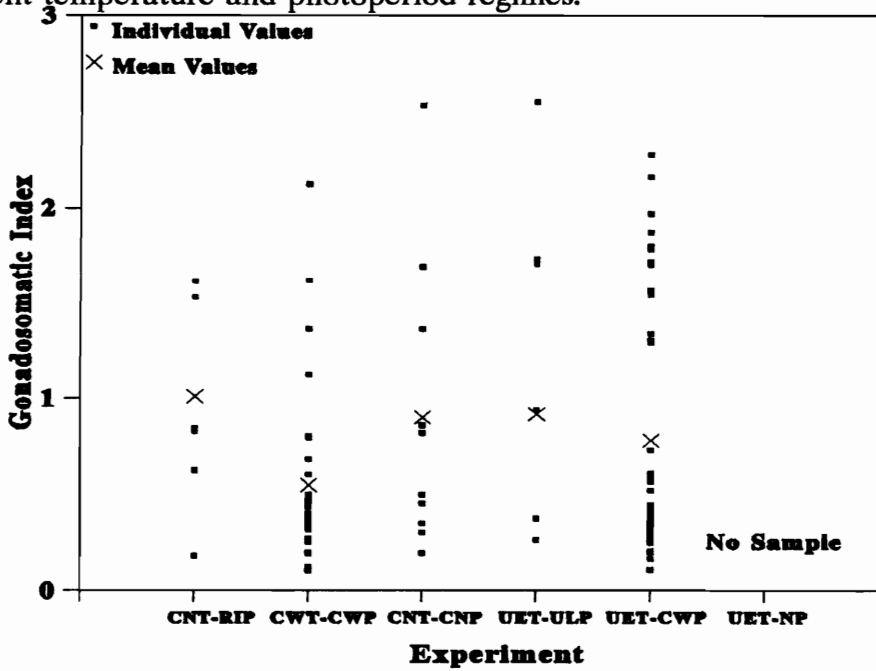
Figure 1.11. Gonadosomatic indices of captive male <u>Cyprinella</u> <u>galactura</u> held under five different temperature and photoperiod regimes.

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Table 1.2. Description and results of experiments to manipulate maturation of the gonads of male <u>Cyprinella</u> <u>galactura</u>.

Trial	Tank type	Trial length (d)	Days[1] from normal season	Mean GSI (p-value[2])	Percent GSI ≥ 0.80 (N)
NT-NP	7000 l oval	Nov to Jul (275)	-43	1.22[3] (0.795)	100 (5)
WF	NA[4]	Nov to Aug (270)	0	1.14[3] (NA)	64 (14)
CNT-RIP	350 l rectangular	Nov to Feb (110)	-109	1.01 (0.675)	67 (7)
UET-ULP	7000 l oval	Aug to Feb (175)	-120	0.92 (0.502)	50 (6)
CNT-CNP	500 l rectangular	Sep to Feb (148)	-123	0.90 (0.419)	50 (10)
UET-CWP	500 l rectangular	Oct to Feb (117)	-123	0.78 (0.098)	30 (46)
CWT-CWP	500 l rectangular	Oct to Feb (117)	-123	0.55 (0.002)	17 (29)

[1]Assumes a 15 June to 15 August spawning season for wild fish; plus or minus is used to indicate days after or before normal season
[2]Probability that the mean GSI value differs from that of wild fish collected in July
[3]July sample only
[4]Not applicable

Aggression among males was observed as temperature and photoperiod approached that of the normal spawning season. Fighting among males in Experiment UET-CWP occurred several weeks prior to that in Experiment CWT-CWP. It is important to note that fighting also occurred among males in other experiments (including several not reported here) as males achieved spawning condition, regardless of

density of fish. Thus, crowding may exacerbate the problem of fighting, but does not cause it.

Aggressiveness of males centered on establishment and protection of territories from other fish, including females. Aggressive males circled and nipped at the tail fins of other fish, rammed other fish broadside, and butted and nipped at the heads of other males. Fish (males and females) lost many scales as a result of fighting and subsequently developed infections. Fighting directly or indirectly eventually caused the deaths of most fish in the tank, as evidenced in preliminary trials. Consequently, fish in the experiments reported here were sampled before excessive mortalities occurred due to aggression of males.

Mean GSI values of females for treatments with compressed temperature cycles (range = 2.31 to 4.19) were statistically smaller than that of wild fish (11.56). $\underline{P}$-values were < .01 for Experiments CNT-CNP and CWT-CWP; a $\underline{p}$-value of .081 for Experiment CNT-RIP was not considered significant, because the mean GSI value was lowest of all experiments and the sample size was one (Table 1.3). Mean GSI values for treatments in which temperature was uniform and elevated throughout the trial, or followed a natural pattern, were not statistically different from values for wild females (range of $\underline{p}$-values = 0.06 to 0.70, and range of mean GSI values = 6.13 to 13.5). The percentage of females that had more than one size of vitellogenic oocytes was positively related to mean GSI values for treatment groups (Table 1.3).

Table 1.3. Description and results of trials to manipulate maturation of the gonads of female Cyprinella galactura.

Trial	Tank type	Trial length (d)	Days[1] from normal season	Mean GSI (p-value[2])	Size (mm) of oocytes[3] (N)	Two[4] oocyte sizes (%)
NT-NP	7000 l oval	Oct to Jul (275)	-43	13.51[5] (0.678)	0.99 (5)	80
WF	NA[6]	Nov to Aug (270)	0	11.56[5] (NA)	1.12 (4)	100
UET-NP	8000 l rectangular	Jun to Oct (122)	+60	9.38 (0.193)	NA (5)	NA
UET-CWP	500 l rectangular	Oct to Feb (117)	-123	7.25 (0.096)	0.78 (13)	54
UET-ULP	7000 l oval	Aug to Feb (175)	-120	6.13 (0.056)	0.88 (5)	40
CNT-CNP	500 l rectangular	Sep to Feb (148)	-123	4.19 (0.001)	0.68 (6)	50
CWT-CWP	500 l rectangular	Oct to Feb (117)	-123	3.78 (0.000)	0.49 (16)	25
CNT-RIP	350 l rectangular	Nov to Feb (110)	-109	2.31 (0.081)	0.33 (1)	0

[1]Assumes a 15 June to 15 August spawning season for wild fish: plus or minus is used to indicate days after or before normal season
[2]Probability that mean GSI value differs from that of wild fish collected in July
[3]Mean diameter for the largest size-class of oocytes at end of experiment
[4]Percent of fish with more than one size class of vitellogenic oocytes in the ovary
[5]July sample only
[6]Not applicable or not available

Description of Gonads and Gametes

The testes and ovaries of whitetail shiners are paired cylindrical organs located below the kidney and attached to the dorsal mesentery in the coelomic cavity. In the testes, a sperm duct arises from the posterior end of each organ, and the pair fuses just posterior to the urinary duct. In the ovaries, an oviduct arises from the posterior end of each organ, and the ducts fuse proximal to the gonopore.

Motile sperm were always present in samples of milt or macerated testes. Motility of the sperm in fresh water was approximately one minute; in phosphate buffered saline, it was > 30 minutes. The heads of the spermatozoa are oval (4.1 ± 0.2 μm long, 2.9 ± 0.1 μm wide), and the tails are 27.6 ± 2.7 μm in length. A pronounced, collar-like midpiece encircles the posterior portion of the head and anterior portion of the tail.

Correlation Between Degree of Tuberculation and GSI Values of Males

Obvious gaps were evident between GSI values for males assigned scores of two, three, or four (Figure 1.12). Based on the position of these gaps and the maximum score for nontuberculated males, a GSI value between 0.80 and 1.25 represents a transition stage from nonspawning to spawning condition for male whitetail shiners. Using the lower value of this range (GSI = 0.80), 80% of category 4, 40% of category 3, 20% of category 2, and 0% of category 1 males were judged to be in or approaching spawning condition. Percent of males in each of the

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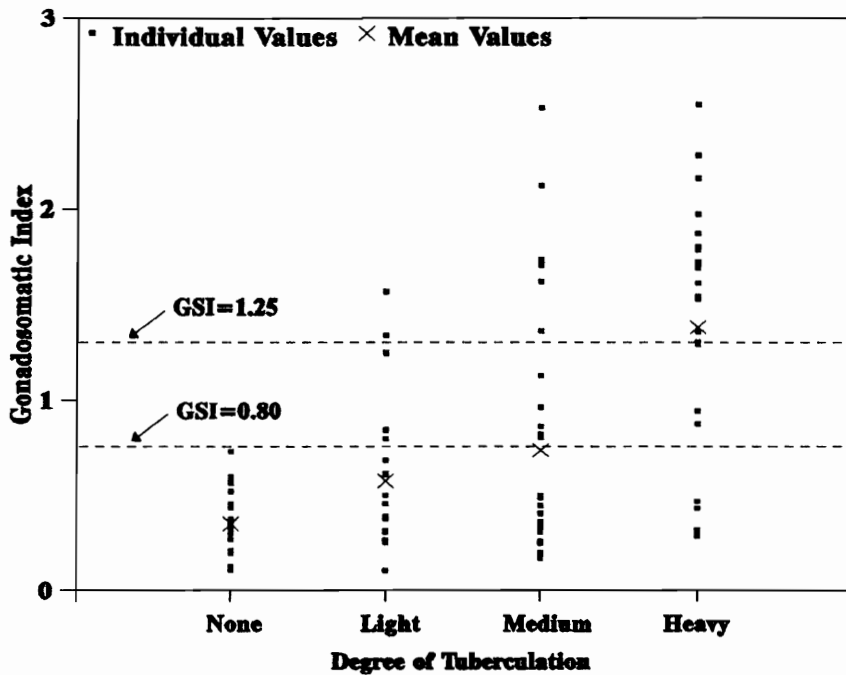
Figure 1.12. Relationship between degree of tuberculation and gonadosomatic index of male Cyprinella galactura.

temperature-photoperiod experiments was also evaluated using the 0.80 GSI value. Seventeen to 100% of the males in the experiments were judged to be potential spawners based on this measure of spawning condition (Table 1.2). More than 50 % of the males had a GSI value $\geq$ 0.80 in all experiments except UET-CWP (30 %) and CWT-CWP (17 %).

Correlations Between Oocyte Diameters and GSI Values of Females

Mean oocyte diameters of the largest size class of oocytes, and the percentages of females with two size classes of vitellogenic oocytes, were positively correlated with

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mean GSI values (R^2 = 0.79 and 0.81, respectively). Wild females had the largest mean oocyte diameter (1.12 mm), and females from Experiment CNT-RIP had the smallest mean diameter of oocytes (0.33 mm). Mean diameters of oocytes sampled from females in all experiments that used a compressed temperature cycle were less than those of females exposed to a simulated natural cycle or to uniform elevated temperature throughout the experiment. The mean GSI values of females in experiments with compressed temperature cycles were significantly different from those of wild females (Table 1.3).

No trends in mean oocyte diameters among core positions of 20 whitetail shiner ovaries were evident from the plotted data (Figure 1.13), and GLM results indicated that mean oocyte diameters were not statistically different among core positions (p=0.10). Therefore, representative samples of oocytes can be collected at any location within the ovaries of the whitetail shiner.

There were moderately high negative correlations between number of oocytes per weight of ovary and mean diameter of oocytes for fish in which oocytes > 0.1 mm were counted (R^2=0.62), and fish in which oocytes > 0.5 mm were counted (R^2=0.56, Figures 1.14 and 1.15). The regression equations were used to estimate fecundity of the whitetail shiner (Figure 1.16). Fecundity estimates for fish with mean oocyte diameters > 0.50 mm are approximately seven-fold lower than those with mean oocyte diameters < 0.50 mm, due to the inclusion of oocytes between 0.10 and 0.50 mm diameter in the counts of the latter. The estimated number of oocytes

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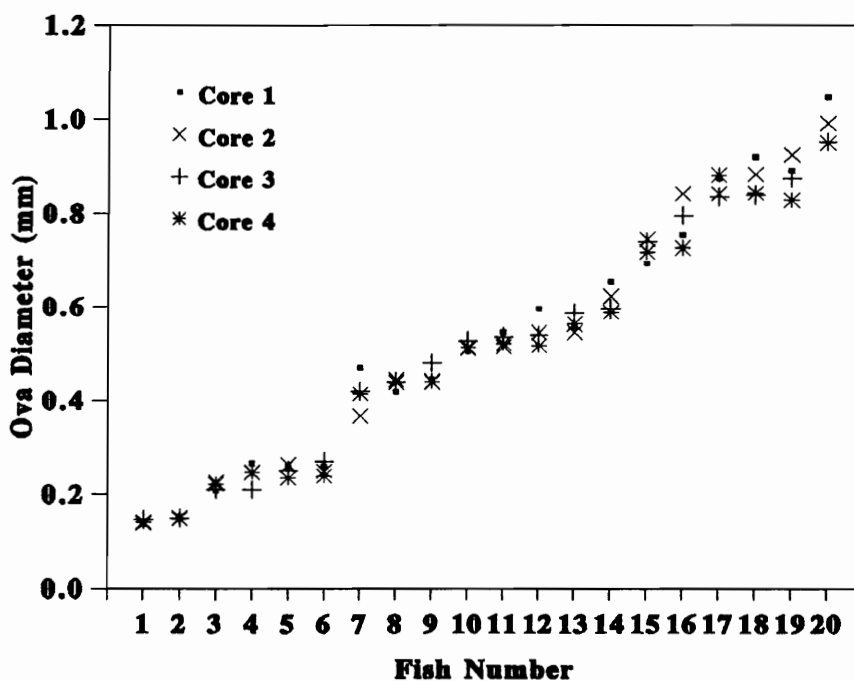
Figure 1.13. Comparison of mean oocyte diameters of four core samples (N=30 for each core) from the ovaries of 20 Cyprinella galactura.
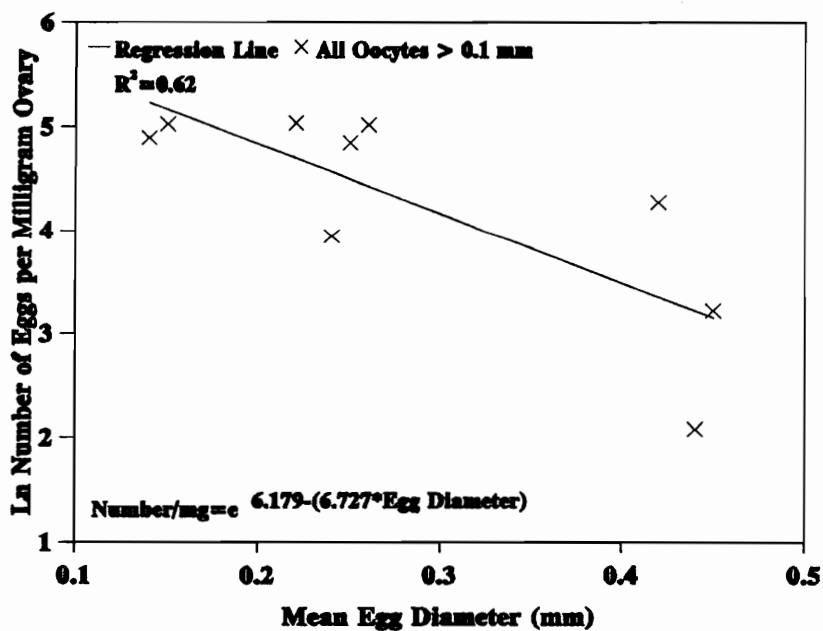
Figure 1.14. Regression of the natural log of the number of Cyprinella galactura oocytes per milligram of ovarian tissue and mean egg diameter.

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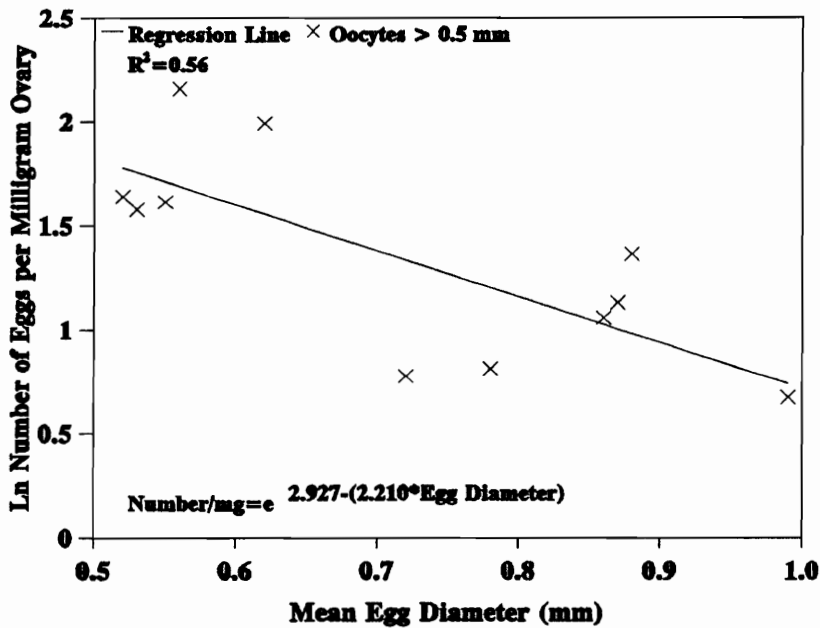
Figure 1.15. Regression of the natural log of the number of <u>Cyprinella</u> <u>galactura</u> oocytes > 0.5 mm per milligram of ovarian tissue against mean oocyte diameter.
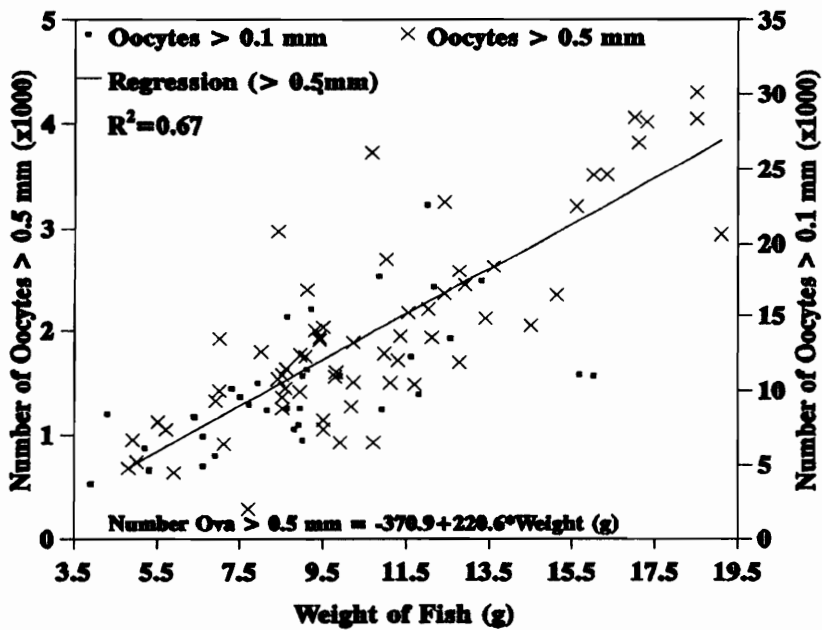
Figure 1.16. Estimates of total fecundity of <u>Cyprinella</u> <u>galactura</u> females based on mean diameter of ova and ovary weight. Regression line is the number of larger vitellogenic oocytes versus weight of female.

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> 0.5 mm ranged from approximately 1000 to 4000 per whitetail shiner, or 180 oocytes per gram of fish (Figure 1.16). The weight-fecundity relationship was: number of oocytes > 0.5 mm = -370.9 + 220.6 * weight (g) (R^2=0.67). The GSI values for the 36 females used to develop the length-weight relationship ranged from 4 to 23, and the regression equation was: ln weight (g) = -13.24 + 3.36 * ln total length (mm) (R^2=0.92). The subsequent length-fecundity relationship was: number of oocytes > 0.5 mm = -5694.9 + 74.6 * total length (mm) (R^2=0.64).

Several trends were evident in the plots of egg size distributions of fish numbers 5 to 20 in Figure 1.13 (Figures 1.17 and 1.18). Ova less than 0.6 mm in diameter dominated in less developed ovaries. In more developed ovaries, a second larger size class of oocytes (approximately 0.6 to 1.0 mm diameter) became evident. In the most developed ovaries, a third and larger size class of ova (approximately 1.0 to 1.4 mm diameter) was observed.

Effects of Isolating Females from Males

Approximately 50 % of the 52 females and 70 % of the 59 male whitetail shiners were judged to be spawners prior to separation of the sexes. Twelve females died during the first isolation period (65 d). Of the 39 that remained, two (5 %) were judged to be in spawning condition. Nine of the ten males examined (90 %) expressed milt when abdominal pressure was applied.
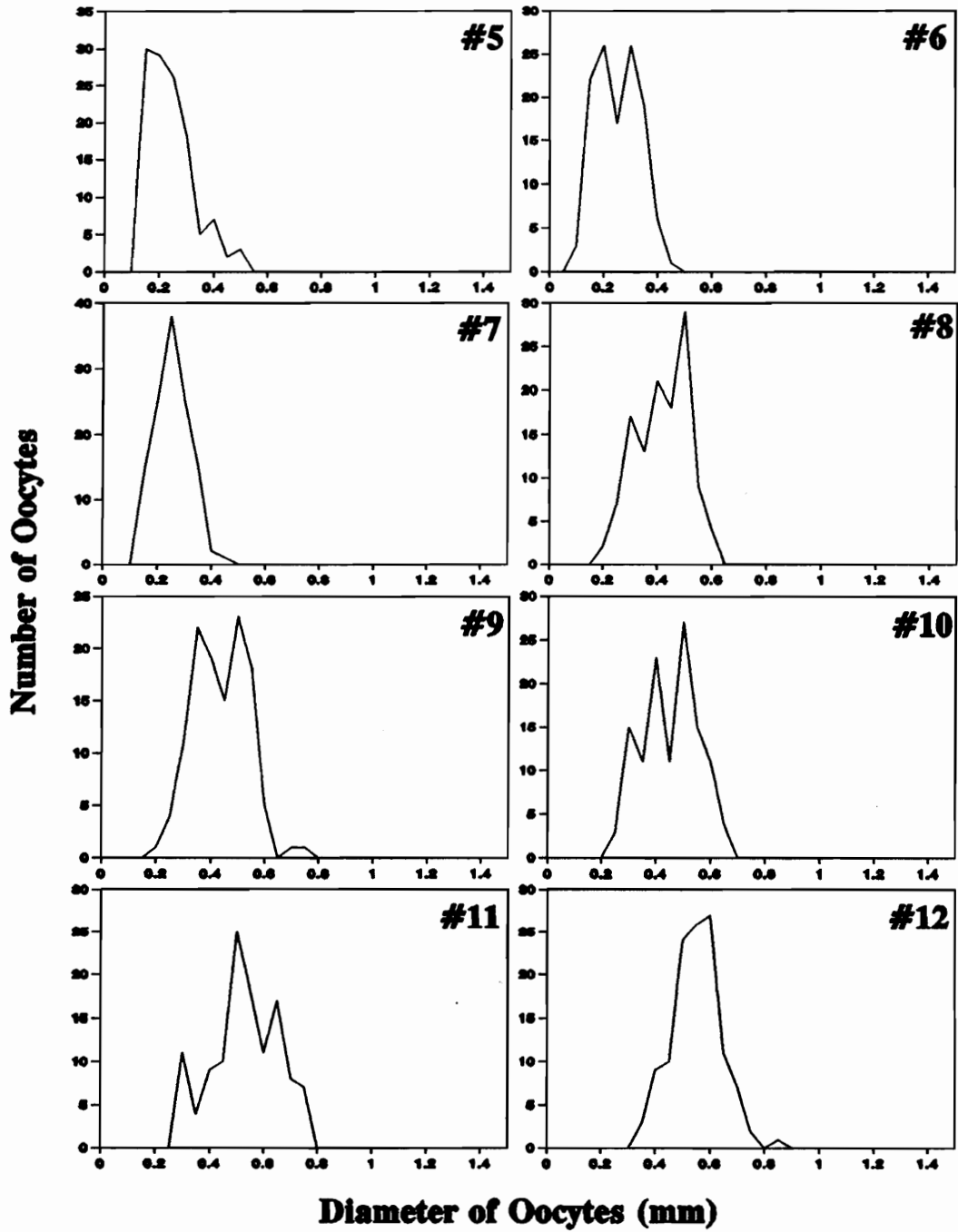
Figure 1.17. Size distributions of 120 ova from female <u>Cyprinella</u> <u>galactura</u>; numbers 5 (top left) to 12 (bottom right) of Figure 1.13.
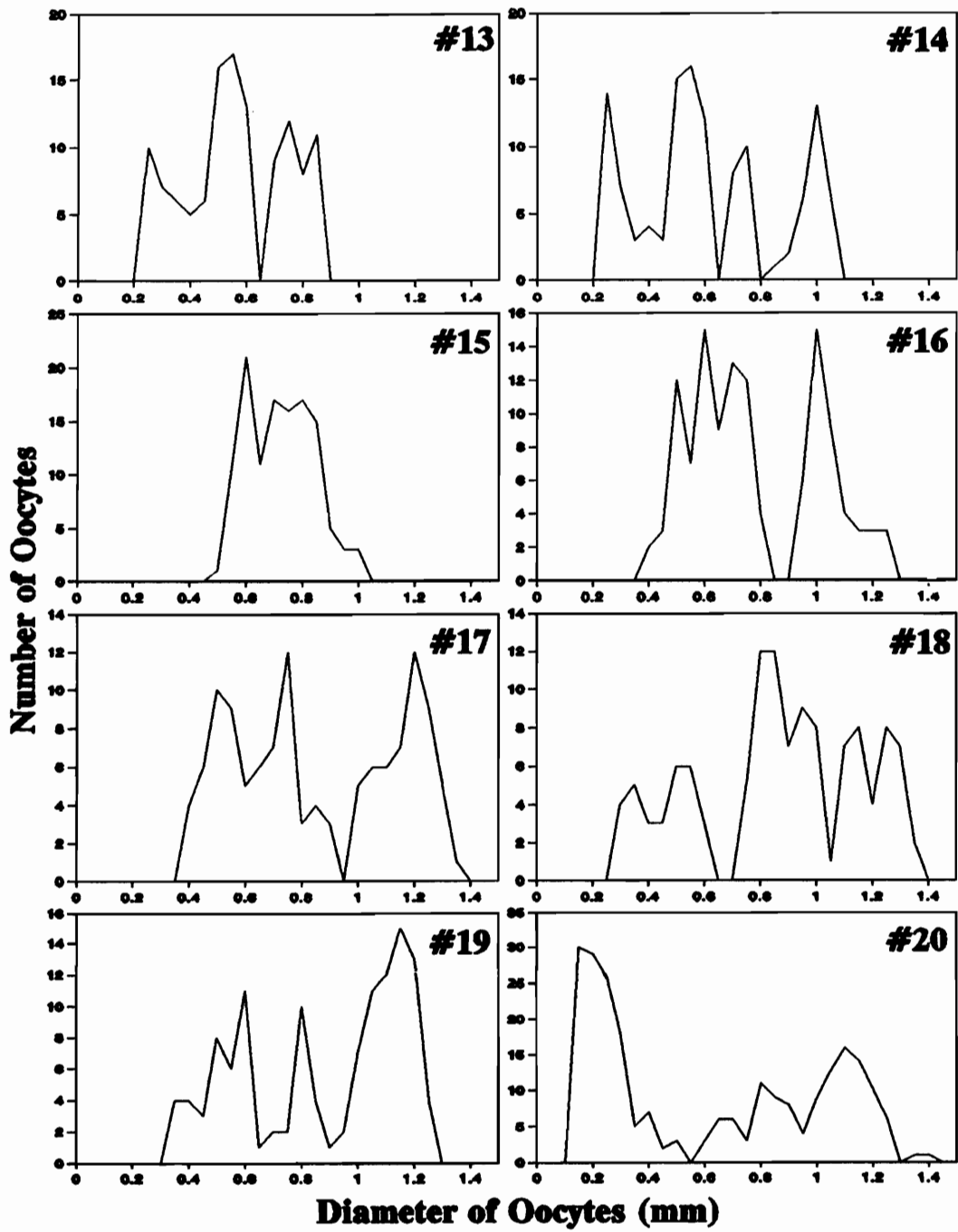
Figure 1.18. Size distributions of 120 ova from female <u>Cyprinella</u> <u>galactura</u>; numbers 13 (top left) to 20 (bottom right) of Figure 1.13.

During the second isolation period, six females in the isolated section and four in the section with males died (75 d). Of the 13 that remained in the section without males, two (15 %) were judged to be in spawning condition and had ova > 0.85 mm in diameter. One of these contained ova in which the germinal vesicle had broken down (i.e., mature ova). Of the 16 that remained in the section with males, 12 (75 %) were judged to be in spawning condition and had ova > 0.85 mm in diameter. Three of these contained ova in which the germinal vesicle had broken down. Large ova could not be obtained from eleven of the females isolated from males or four of the females held with males. The mean condition factor of the isolated females (0.79) was slightly higher than that of the females held with males (0.75), but this difference was not significant ($p=0.13$).

Maturation of Fish Captured in Mid-Winter

All fish collected during the winter achieved spawning condition in captivity between day 250 (May) and day 310 (July) of Trial NT-NP. Maximum ova diameters of the females ranged from 0.95 to 1.75 mm, and the average diameter of larger ova for all females was 1.12 mm. Nineteen females and 18 males received hormonal injections, and five of the females produced viable spawns.

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Maturation of Captive Fish at Constant Temperature and Photoperiod

Fifty-four days following the separation of spawners from non-spawners, seven females and no males in the nonspawner section were judged to be in spawning condition. Eight fish in the spawner section were no longer in spawning condition. By day 96, one additional female and three males in the nonspawner section were judged to be in spawning condition. In the spawner section, five females and three males were not in spawning condition. By day 175, all fish except one male had achieved spawning condition in the non-spawner section.

Development of New Complements of Mature Ova in Females

Three post-spawning experiment females had mature ova less than 25 d after receiving hormonal injections. Atretic ova were found in several of the females. All three of the females isolated in aquaria had mature ova by 27 d. The maximum ova diameters ranged from 1.05 to 1.25 mm, and the mean diameter of all larger ova sampled from the three females was 1.10 mm.

Discussion

This discussion is divided into four sections that incorporate information derived from my experiments and observations, and from the literature. Information on the biology of species is generalized to the generic level where deemed appropriate. The discussion is couched in the context of developing techniques to induce and maintain spawning condition of rare Cyprinella spp., as part of a controlled propagation program. The first two sections focus on the biological characteristics of the gonads of Cyprinella spp.; the third, on effects of environmental manipulation on gonadal manipulation; and the fourth, on development of strategies to induce and maintain gonadal maturation of rare Cyprinella spp.

Structure and Function of the Testes

The gross morphology of the testes of the whitetail shiner is similar to that of most other teleosts. They are paired, smooth, elongate, cylindrical organs attached to the dorsal body wall (Nagahama 1983). A main sperm duct arises from the posterior surface of each organ. They fuse just anterior to the urogenital papilla and immediately join with the urinary duct. Spermatozoa exit the body via the urogenital pore.

The general morphology of the testes of the whitetail shiner implies that they are similar in function to those of other cyprinids (i.e., the common carp and the goldfish, Carassius auratus). The lobular type of testis in these cyprinids has a central

lumen that receives spermatozoa when released from cysts. The cysts remain stationary along the lobule during spermatogenesis (Billard et al. 1982). An important aspect of previously described cyprinid testes is the presence of germ cell types throughout the year that allow for nearly continuous production of viable sperm. Studies of the endocrine patterns of cyprinids suggest that the gonadotropin involved is controlled by external (mainly temperature but also photoperiod) and gonadal factors (Billard et al. 1982). Because testes and spermatozoa of other Cyprinella spp. have not been described, comparisons are made with those of the common carp and the goldfish.

In the common carp, males kept at temperatures above 10 C produce fertile sperm at any season, provided they receive gonadotropin injections at temperatures less than 18 C (Billard 1990). In the goldfish, males maintained maturity through the year when held at 13 to 14 C, and released milt within a day when moved to aquaria at 20 C (Yamamoto et al. 1966). However, too high a temperature (24 C) can result in cyclic or irregular milt production in goldfish (Hanyu and Razani 1985).

Motile sperm were observed when water was added to macerated sections of testes dissected from whitetail shiner males; even in males with weakly developed secondary sexual characteristics. This is similar to observations for common carp males. Histological examination of common carp testes shows that spermatozoa, although reduced in number during the nonspawning season, are present year-round

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(Billard 1990). When examined under a light microscope, milt obtained by squeezing the abdomen of whitetail shiner males always contained numerous motile sperm.

Spermatozoa of the whitetail shiner are similar in appearance, but slightly larger than those of the common carp. The heads of whitetail shiner spermatozoa averaged 2.9 ± 0.1 μm wide by 4.1 ± 0.2 μm long, and the tails averaged 27.6 ± 2.7 μm in length. The heads of common carp spermatozoa are 2.0 to 2.5 μm wide by 3.3 μm long, and the tails are 40 to 60 μm in length (Billard et al. 1986). The heads of goldfish spermatozoa are spherical (3.2 μm in diameter), and the entire length of a spermatozoan is $\approx$ 60 μm (Fribourgh et al. 1970). Thus, the tails of whitetail shiner spermatozoa are appreciably shorter than that of either the common carp or the goldfish.

Duration of motility of common carp sperm in fresh or saline water does not exceed 40 to 60 s (Billard et al. 1986). The duration of motility of whitetail shiner spermatozoa was approximately one minute in fresh water, but exceeded 30 min (albeit motility was much reduced) in phosphate buffered saline. A very pronounced collar-like midpiece in whitetail shiner spermatozoa may account for the longer duration in motility, because the midpiece contains the mitochondria (Fribourgh et al. 1970).

Procurement of sperm from male Cyprinella spp. for artificial propagation is seemingly feasible, because mature spermatozoa were present even in nonspawners. More importantly, milt can usually be obtained from highly colored males by gently

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squeezing the abdomen. This precludes the need to sacrifice males, which is a prime consideration when dealing with rare fishes. Injections of hormones seems to enhance production of free-flowing milt, and is addressed more thoroughly in Chapter 2.

Structure and Function of the Ovaries

The external morphology of the ovaries of whitetail shiners is similar to that of most other teleosts; paired, cylindrical, sac-like organs. The ovary is asynchronous, meaning it contains oocytes at all stages of development during the spawning season (Wallace and Selman 1981). Mature oocytes are released during ovulation into the ovarian cavity, which is continuous with the oviduct (Nagahama 1983). Oocytes then migrate through the oviducts which converge near the gonopore, and exit via the gonopore at time of spawning.

Sampling of unovulated oocytes to assess state of development in whitetail shiners, and presumably in other Cyprinella spp., is not influenced by position of oocytes within the ovary. However, oocytes are not uniformly distributed when ovulated oocytes are present because they are concentrated in the posterior portion of ovaries (Gale 1986, Heins and Rabito 1986, Ferguson 1990). I did not examine the ovaries of recently ovulated whitetail shiner females, but a gross evaluation of the ovaries should be sufficient to determine whether they contain ovulated oocytes. Ova in Cyprinella spp. are mature at a size of ≈ 1.0 mm in diameter, and GSI values for

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Table 1.4. Comparison of reproductive characteristics of <u>Cyprinella</u> spp. females.

Species of <u>Cyprinella</u>	Clutch sizes of (mature) or spawned ova	Diameter (mm) of (mature) or spawned ova	Gonadoso-matic index (%) of wild fish	Reference
<u>analostana</u>	6-634	1.5[1,2]	(≈9.5)[1]	Gale and Buynak 1978
<u>caerulea</u>	---[3]	---	≈9.0-14.0[1]	Krotzer 1990
<u>callistia</u>	(44-570)	(0.9-1.4)[4]	6.8-11.1[1]	Ferguson 1990
<u>callitaenia</u>	(88-230)	(0.9-1.3) 1.2-1.7[2]	≈5.0-7.0[1]	Wallace & Ramsey 1981
<u>galactura</u>	(404-1815) 127-382	(1.0-1.6)	---	Outten 1958
<u>galactura</u>	20-1650[5]	(0.8-1.6) 1.7-2.0[1]	10.1-11.6[1]	This study
<u>leedsi</u>	(26-228)	(0.8-1.1)	---	Heins & Rabito 1986
<u>lutrensis</u>	---	1.0-1.3	≈6.0-11.0[1]	Farringer et al. 1979
<u>lutrensis</u>	131-1661	(0.9-1.2) 0.9-1.1[6]	---	Gale 1986
<u>monacha</u>	(157-791)	(0.8-1.4)	12.5-14.7	Jenkins & Burkhead 1984
<u>nivea</u>	(112-545)	(1.2-1.6)	≈12.0[1]	Cloutman & Harrell 1987
<u>spiloptera</u>	156-986	---	---	Gale & Gale 1977
<u>venusta</u>	(140-457)	(0.9-1.2)[1]	6.3-12.1[1]	Heins & Baker 1987
<u>venusta</u>	(140-457)	(0.8-1.4)[1]	5.8-19.1	Heins & Dorsett 1986
<u>whipplei</u>	---	1.5[1,2]	---	Hankison 1930

[1]Mean value of sample(s)
[2]Assumed to be diameter of chorion
[3]Not determined
[4]Includes diameter of ovulated oocytes
[5]From hormonally injected fish
[6]Assumed to be diameter of yolk

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females during the spawning season are generally greater than 6 (Table 1.4).

Size of ovulated oocytes should be distinguished from unovulated, mature oocytes in descriptions of ovarian development, because ovulated oocytes are enlarged. Enlargement occurs as a result of hydration of the ovary brought on by the surge of gonadotropin at the time of ovulation (Clemens and Grant 1964). For example, oocytes of the mummichog, Fundulus heteroclitus, enlarge from ≈ 1.3 to ≈ 1.8 mm (Wallace and Selman 1978), and those of the common carp enlarge from ≈ 1.0 to ≈ 1.2 mm in diameter as a result of hydration (Horvath 1986). Heins and Rabito (1986) found that oocytes of the bannerfin shiner, Cyprinella leedsi, ranged from 1.3 to 1.6 mm in diameter, whereas unovulated but mature oocytes ranged from 0.8 to 1.1 mm in diameter. In hormonally-injected whitetail shiner females, oocytes increased from a mean size of 1.12 mm to 1.19 mm as they matured (see Chapter 2). Thus, oocytes of Cyprinella spp. increased approximately 6.3 % in diameter as a result of hydration during the maturation process. Additional hydration may occur during the ovulation process, but this was not determined.

Fecundity is the number of ripening oocytes and mature ova or eggs just prior to spawning, and represents spawning potential, usually for the whole season (Snyder 1983). Thus, seasonal fecundity of fish with synchronous ovaries can be determined simply by counting the number of vitellogenic oocytes in the ovaries prior to the spawning season. Determination of seasonal fecundity of a species with asynchronous ovaries is more difficult. Heins and Dorsett (1986) equated fecundity with clutch size

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for the blacktail shiner, Cyprinella venusta, where clutch size was defined as number of mature, unovulated oocytes in the ovaries. Obviously, this measure of fecundity does not begin to approach the potential seasonal fecundity for the species. Consequently, I would discourage use of the term fecundity in this context and simply replace it with 'number of mature oocytes'. I would also caution against referring to number of mature oocytes as clutch size, unless it is defined as 'clutch size of mature ova', to distinguish it from clutch size of spawned ova, because that is the traditional use of the term. The two clutch sizes are not necessarily equivalent. For example, Outten (1958) determined that the clutch size of mature oocytes in whitetail shiners ranged from 404 to 1815 mature oocytes, whereas that of spawned ova ranged from 127 to 382 ova.

Seasonal fecundity has only been determined for the red shiner, and it ranged from 4,701 to 8,248 ova per female (Gale 1986). The seasonal fecundity seemingly is a function of length of the spawning season, inasmuch as two red shiner females, maintained at constant light and warm temperature, spawned continuously for 21 months, and produced 349 clutches with 221,000 ova (Gale 1986). By spawning repeatedly, female Cyprinella spp. can produce a volume of eggs greater than the volume of their body in a single spawning season; females of the satinfin shiner have produced an ova volume ≤ 1.7 times volume of body and those of the red shiner have produced an ova volume ≤ 2.5 times volume of body (Gale and Buynak 1978, Gale 1986). My estimates of fecundity of larger vitellogenic oocytes (1000 to 4000 per

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female) very likely do not represent the seasonal potential of the species, and highlight the difficulty in using a single ovarian measurement to estimate fecundity of species that develop and spawn multiple clutches during the spawning season.

In general, it seems that Cyprinella spp. contain two size classes of vitellogenic oocytes during the spawning season, exclusive of ovulated oocytes; the whitetail shiner (< 0.75 and 0.75 to 1.4 mm), the bannerfin shiner (< 0.8 and 0.8 to 1.1 mm), the spotfin chub, Cyprinella monacha (< 0.8 and 0.8 to 1.4 mm), and the red shiner (< 0.9 and 0.9 to 1.2 mm) (Jenkins and Burkhead 1984, Gale 1986, Heins and Rabito 1986). Gale (1986), based on his examination of the ovaries of red shiners, concluded that some previtellogenic oocytes (0.05 to 0.3 mm) mature and are spawned in less than a week. Thus, fecundity of Cyprinella spp., is best described as indeterminate (Gale 1986). A positive relationship between seasonal fecundity and degree-days may exist for Cyprinella spp., but it has not been described.

Environmental Control of Maturation

Based on my findings, captive whitetail shiner males and females mature out-of-season under a variety of photoperiod and temperature regimes. Changes in temperature are not requisite to maturation of gonads of whitetail shiners collected after the natural spawning season. Harrington (1950) found that the bridle shiner, Notropis bifrenatus, also did not require a change in photoperiod or temperature to achieve spawning condition and spawn. Fish collected in midwinter and exposed to

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constant temperature (19.7 C) for 72 d and constant photoperiod (17 h) for 41 d matured and spawned. However, Harrington (1957) noted that bridle shiners, captured just after the spawning season, took 97 d to mature and spawn when subjected to similar environmental conditions. Based on an examination of gonads during the experiment, he concluded that a refractory period for gonadal development of approximately one month exists for the bridle shiner.

Gonadal maturation of captive whitetail shiners was correlated with elevated temperatures in all experiments, and constant, as compared to changing temperature, seems to hasten development. Hence, warm water ($\geq$ 20 C) is required for gonadal maturation, and continuous warm temperature seems to hasten the development. Too high a temperature suppresses gonadal activity in some temperate fishes. For example, Hubbs and Bailey (1977) concluded that termination of the breeding season of the inland silverside, Menidia beryllina, was associated with high temperature ($\geq$ 31 C). De Vlaming and Paquette (1977) reported that under 16 h of light, gonadal regression of golden shiners, Notemigonus crysoleucas, occurred at 27 C, but not at 25 C. Depressions in gonadal activity of red shiners and blacktail shiners have been correlated with dry, hot weather (Farringer et al. 1979; Lehtinen and Echelle 1979). Gale (1986) observed that spawning of red shiners was interrupted above 34 C in aquaria. It is likely that other Cyprinella spp. also have thermal shut-off mechanisms regulating gonadal development, but the temperatures seem to be

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relatively high. For example Outten (1958) observed whitetail shiners spawning at 28 C, and the red shiner readily spawns at 34 C (Gale 1986).

The effects of photoperiod on maturation are less clear. Maturation of fish in experiments with changing photoperiod was associated with longer photoperiod, but de Vlaming and Paquette (1977) concluded that short photoperiod (9 h) suppresses gonadal activity even if water temperature is high. Fish in Experiment UET-NP retained gonadal maturity at a photoperiod of ≈ 12.4 h of light. Pflieger (1965) found that spotfin shiners, Cyprinella spiloptera, and steelcolor shiners, Cyprinella whipplei, will mature out-of-season when they are held at warm temperatures (21-24 C) and either constant long photoperiod (16 h light) or natural photoperiod. My captive fish exposed to natural photoperiod were mature by 9 March, and day length at that time would have been approximately 11.7 h. Thus, a relatively short photoperiod seemingly permits gonadal development to proceed in Cyprinella spp. The results of Pflieger's (1965) experiment and my Experiment UET-NP indicate that 12.5 h light is sufficient for development and retention of reproductive condition for Cyprinella spp.

Although changes in photoperiod are not requisite for gonadal development, bringing fish through a photoperiod cycle may help to synchronize maturation of gonads among females. Fifty percent or greater of females exposed to changing photoperiod had two sizes of vitellogenic oocytes, whereas only 40 % of fish in Experiment UET-ULP had two classes of vitellogenic oocytes.

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Maintenance of spawning condition in Cyprinella spp. captured during the natural spawning season is a more efficacious strategy, from the perspective of controlled propagation, than bringing out-of-season fish into spawning condition, because time is not lost waiting for gametes to mature. Fish in Experiment UET-NP were kept in spawning condition from June to mid-October simply by maintaining them at an elevated temperature of 22 C. Other fish, used in spawning experiments and held in the oval raceway at 22 C and 16 h light, also retained spawning condition past the normal spawning season. For example, 15 males and 15 females, examined for spawning condition on 10 December, were all judged to be spawners. Milt could be obtained from most males, and nearly all females had distended abdomens. Ovulated but overripe ova were obtained from one female. In general, once fish became spawners and were used in spawning trials, they tended to stay in spawning condition and were used repeatedly.

De Vlaming and Shing (1977) concluded that golden shiners cannot maintain reproductive condition indefinitely, inasmuch as no fish held under spawning conditions were spawners after 156 d. They attributed the cessation in spawning to reproductive fatigue. The length of time that Cyprinella spp. can maintain spawning condition is unknown. However, it apparently is longer than that of the golden shiner. Gale (1986) found that red shiners, exposed to constant long photoperiod and warm temperature, essentially spawned continuously for two years. Although natural spawning did not readily occur, a similar condition was observed for whitetail shiners

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held under similar conditions, inasmuch as there were always some fish in reproductive condition over approximately two years.

Injections of hormones used in spawning experiments may have enhanced gonadal development, because a surge of gonadotropin can initiate or enhance vitellogenesis. Campbell and Idler (1976) showed that vitellogenesis in the winter flounder, Pleuronectes americanus, is dependent upon a nonglycoprotein gonadotropin. In the threespine stickleback, Gasterosteus aculeatus, and the fourspine stickleback, Apeltes quadracus, new clutches of primordial oocytes are recruited to the vitellogenic and maturation stage by the surge of gonadotropin that occurs at the time of ovulation-spawning (Wallace and Selman 1979). Several nonspawners held for weeks in the oval raceway were given injections of LHRHa and domperidone, and within several weeks most became spawners. However, without controls no conclusions on the effects of hormonal injections on inducing maturation of nonspawners is possible. A similar mechanism for Cyprinella spp. would explain the tendency for fish used in spawning experiments or those that spawned naturally to retain spawning condition.

Recommendations for Future Work

The reproductive biology of all species of Cyprinella seems to be similar, as well as the response of captive male and female gonads to environmental parameters. Males apparently have some mature sperm year round, and more importantly, can

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be kept or brought into spawning condition simply by exposing them to water temperature ≥ 20 C and long photoperiod.

A difference among species that needs to be addressed is the degree of aggressiveness and skittishness that manifests itself during spawning conditions. For example, whitetail shiner males were very aggressive toward each other as well as females, even when cover was provided. Similarly, Mark Ferguson (personal communication, Virginia Department of Game and Inland Fisheries) noted distinct differences in aggressiveness and skittishness of five species of Cyprinella that were held under identical conditions. Alabama shiners, Cyprinella callistia, and greenfin shiners, Cyprinella chloristia, became very agitated when approached, frequently ramming headlong into the side of the tank. He found the Tallapoosa shiner, Cyprinella gibbsi, the tricolor shiner, Cyprinella trichroistia, and the fieryblack shiner, Cyprinella pyrrhomelas, much more amenable to captivity in aquaria. Consequently, rare Cyprinella spp. brought into the laboratory should be observed to ensure that holding conditions are suitable. If males are overly aggressive or fish are too skittish, mortalities can be reduced by moving fish to larger tanks. The only tank in which I was able to maintain whitetail shiners in spawning condition was the 7000 L oval raceway. They were held in screened sections of either 1.2 or 2.4 m in length and 1.2 m in width. Mortalities were notably higher in Living Streams (usable area of 1.4 X 0.5 m) and aquaria (110 L). Depth was approximately the same in the oval raceway and the Living Streams (≈ 0.4 m). Thus, the additional width seemingly

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allowed enough room for less aggressive fish to sequester themselves from more aggressive fish. Living Streams, although unsuccessful in holding the relatively large number of fish used in my experiments, may suffice for holding smaller numbers of fish. Another option is to isolate males in breeding condition, but the effects of isolation on stress has not been determined. In addition, use of too small a tank likely will inordinately elevate stress of the more easily stressed species.

Based on my observations of spotfin chubs in the wild, it is likely that they tend toward the more aggressive and skittish end of the scale of Cyprinella spp. Males and females were more difficult to approach than whitetail shiners while snorkeling, and breeding males (apparently guarding spawning territories over slabs of bedrock) were always found alone.

Gonads of female Cyprinella spp., as with males, will mature under long photoperiod and warm water (defined hereafter as ≥ 20 C) conditions. Based on current knowledge, there are two strategies that I would recommend to optimize production of ova for captive propagation and to minimize stress to wild populations. The first is to capture wild females at the end of the natural spawning season and to maintain them in spawning condition by providing long photoperiod (≥ 16 h light) and warm water. Collections of rare fish should not be made at temperatures > 24 C in order to avoid undue handling stress. Ideally, collections should be made at temperatures < 20 C to further reduce negative effects of handling stress at warm temperatures (potential stress from low oxygen or high ammonia, greater mechanical

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damage, increased excitability, etc.). Fish should be prophylactically treated for disease immediately after capture and periodically thereafter; every two to three weeks is not too often. These fish can be spawned in the off season and then released at the beginning of the next natural spawning season.

The second strategy is to collect larger juveniles during the fall, again when water temperatures are < 20 C. These fish should then be held at a warm water temperature and long photoperiod ($\geq$ 16 h light) to encourage precocial development of gonads. They can be fed a base diet of commercial flake food, supplemented with either live food or moist pellet. Several spawns could be obtained from these fish prior to the start of the natural spawning season, and then the fish could be returned to their natural population, as adults, prior to the spawning season. Either of the above two methods would minimize the impact of removal of adults from depauperate wild populations.

Captive propagation, using either of the above strategies, would benefit from optimization of egg production. I suggest research in the following areas: 1) enhancement of rate of maturation of out-of-season fish by injection of hormones; 2) evaluation of the effects of temperature on number of ova produced and clutch size; 3) effect of constant light versus long light (e.g., 16 h) on egg production; and 4) evaluation of the effects of diet on egg production.

Females captured near the end of the spawning season may or may not be in spawning condition. Two factors that should be addressed are whether a refractive

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period exists for post-spawning females and whether hormonal injection (i.e., a surge of gonadotropin) can advance reproductive condition of post-spawning females, regardless of a potential refractory period. The effects of hormonal injections on juveniles, once they reach reproductive size, should also be investigated. Successful application of hormonal therapy would increase the number of females that can be used as brood stock, or decrease the time required to gain (regain) spawning condition.

The effects of temperature on the ovarian process in Cyprinella spp. are poorly understood. Time between spawnings decreases as temperature increases, but clutch sizes are smaller. Too high of a water temperature can inhibit egg production. This problem can be precluded by not holding captive Cyprinella spp. at temperatures near the upper range of reported spawning temperatures for these species. From the perspective of controlled propagation, larger less frequently spawned clutches are more valuable than smaller more frequently spawned clutches. Larger clutches facilitate rearing efforts by providing more larvae of uniform age and size. Thus, experiments to determine the effects of various temperature regimes on frequency of spawning and clutch size are desirable.

Water temperature can also affect total egg production. For example, Gale (1986) found that red shiners produced 16% more ova at warmer temperatures. Maximum GSI values reported for bluestripe shiner, Cyprinella callitaenia, the blue shiner, Cyprinella caerulea, the whitefin shiner, Cyprinella nivea, and the blacktail

shiner occur between 21 and 25 C for wild populations (Wallace and Ramsey 1981, Heins and Dorsett 1986, Cloutman and Harrell 1987, Krotzer 1990). Temperatures at which red shiners will spawn in aquaria range from $\approx$ 20 to 34 C (Gale 1986), but temperatures reported during the natural spawning season for other Cyprinella spp. are slightly lower and range from 20 to 29 C (Outten 1958, Minckley 1972, Wallace and Ramsey 1981, Heins and Dorsett 1986, Cloutman and Harrell 1987, Ferguson 1990, Krotzer 1990). These temperatures provide a range to study the effects of temperature on egg production. Optimization of the balance between egg production and clutch size should be a prime consideration. Based on current knowledge, rare fishes should be held at the lower end of the temperature range for maximum GSI values ($\approx$ 21 to 23 C). This would encourage production of larger clutches, and reduce handling stress for those fishes which must be hormonally injected to produce consistent spawns. Effects of decreasing photoperiod on gonadal regression of Cyprinella spp. are not known, and were not evaluated in this study. Research in this area is deemed unimportant from the perspective of controlled propagation, because fish can apparently be maintained indefinitely in reproductive condition simply by providing constant long photoperiod and warm temperature. However, the effects of constant versus long light conditions should be investigated. Absence of a rest period (e.g., 8 h dark) may increase stress and reduce egg production, particularly for more skittish species. Consequently, 16 h light is recommended for holding rare Cyprinella spp., until additional studies are conducted. Changes in photoperiod may

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help synchronize gonadal maturation of fish, but this is not considered necessary. I suggest focusing research efforts on refinement of methods to hasten development of reproductive condition of juveniles and out-of-season adults.

A base diet of flake food is recommended for rare Cyprinella spp., because it is readily accepted by other Cyprinella spp. (Heins and Rabito 1986, Mark Ferguson personal communication, Virginia Department of Fisheries and Wildlife Sciences). Use of live food obtained from the wild is discouraged because of the potential for disease introduction. A base diet of cultured live food is also discouraged, because live cultures take considerable time to maintain and the supply of these foods is much less dependable than that of prepared foods. However, the use of other prepared diets or cultured live foods is recommended as a supplement to help ensure an adequate diet for captive fish. Effects of diet and feeding regime on egg production need to be evaluated.

CHAPTER 2

Controlled spawning of <u>Cyprinella</u> <u>galactura</u>

Introduction

The goal of this research was to develop a protocol for spawning rare <u>Cyprinella</u> spp. Several species of <u>Cyprinella</u> readily spawn under controlled conditions (Gale and Gale 1977, Gale and Buynak 1978, Gale 1986, Heins and Rabito 1986). One objective of this research was to synthesize information on methods used to spawn <u>Cyprinella</u> spp. in captivity and evaluate them in experiments using the whitetail shiner, <u>Cyprinella</u> <u>galactura</u>, as a model species. A second objective was to evaluate the use of hormonal injections as a method to promote gonadal maturation, or induce ovulation and spawning of <u>Cyprinella</u> spp. using the whitetail shiner as a model species. The knowledge obtained from all experiments and information garnered from the literature was used to develop a protocol for spawning rare <u>Cyprinella</u> spp.

Two general types of spawning experiments are referenced in the following text. The first experiments were natural tank-spawning tests, and they used no hormonal injections or low dose hormonal injections to promote gonadal maturation and spawning behavior of whitetail shiners. The second experiments were induced-

spawning tests. They used injections of high doses of hormones to promote ovulation and induce the spawning of whitetail shiners.

Number of fish that spawned was the primary criterion used to evaluate the effectiveness of all treatments. Other key criteria used to evaluate the efficacy of hormonal treatments were changes in the position of the germinal vesicle and diameter of oocytes, because they have proven valuable for other species (Conte et al. 1988, Rottmann and Shireman 1988).

Several characteristics of spawned ova deemed relevant to development of a spawning protocol for Cyprinella spp. were also evaluated. The effectiveness of dry-stripping versus wet-stripping methods for fertilizing ova from whitetail shiner females was compared. Other factors critically examined were percent fertilization of ova as a function of source of fish, methods to hatch embryos, time to hatch, and the percent larvae recovered from spawned eggs.

The organization of this chapter is by subsections that concentrate on tank-spawning experiments, induced-spawning experiments, and characteristics of spawned ova. The discussion also includes a subsection with a suggested protocol for spawning rare Cyprinella spp. and addresses topics in need of further research, relative to spawning rare Cyprinella spp. The discussion is couched in terms of working with rare fishes, and generalizations to all species of Cyprinella are made where appropriate.

Materials and Methods

Fish used in all experiments were captured during the spawning season (late June to early August) by nightly seining of sandy shorelines of the New River, Montgomery County, Virginia, or by electrofishing in two tributaries of the New River; Wolf Creek, Bland County and Toms Creek, Montgomery County. Shortly after capture, fish were prophylactically treated for disease for at least 4 h with salt (0.2 %) and Furacin (5 to 10 mg/L), and with formalin for at least 4 h (25 mg/L) or for 1 h (250 mg/L). They were treated periodically thereafter.

Tank-Spawning Experiments

Two types of tanks were used in tank-spawning experiments. One type of tank was a circular, metal tank (1.8 m diameter X 0.6 m height). Two of these tanks were used, and both were kept outdoors. Consequently, fish in these tanks were exposed to natural temperature and photoperiod regimes. Tank bottoms were covered with pea size gravel, and submersible pumps created a circular current within the tanks. Cover consisted of large concrete blocks and half-sections of 51 mm diameter PVC pipe that were 76 mm or 102 mm long. Ceramic tile (102 mm X 102 mm) spawning substrates with 3 mm and 6 mm crevices, similar to those used by Gale and Buynak (1978), Rabito and Heins (1985), and Gale (1986) for tank-spawning other Cyprinella spp., were perched off the bottom atop 50 mm long sections of 38 mm diameter PVC pipe.

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The other type of tank used was a 110 L aquarium. Four indoor units, consisting of five aquaria each, were used in separate trials. Each unit drained into a 175 L undergravel biofilter and received a steady, small input of activated charcoal-filtered municipal water. Water from the biofilter was pumped to a head tank and recirculated to aquaria. Each aquarium was supplied with two air stones and the same type of spawning substrates used in outdoor trials. Sides of the tanks were not covered to prevent disturbance of tanks, because preliminary trials indicated that schooling behavior was suppressed, and skittishness of fish in enclosed tanks was notably greater than that of fish in uncovered tanks. Rabito and Heins (1985) reported that placement of cardboard blinds around tanks that contained the bannerfin shiner, Cyprinella leedsi, suppressed schooling and mating behavior. Air flow was adjusted to provide a moderately strong circulation of water within aquaria. Water temperature in each unit was controlled by a thermostat and 220 volt heating element. Temperature in one study was held constant at 25 C, and in the others it was gradually elevated from 25 to 28 C, 26 to 28 C, or 24 to 28 C. Fish were exposed to natural photoperiod through translucent ceiling panels.

Heins and Rabito (1986) and Mark Ferguson (personal communication, Virginia Department of Game and Inland Fisheries) successfully used commercial flake food to maintain Cyprinella spp. in breeding condition. Preliminary trials indicated that whitetail shiners also readily accepts commercial flake food, as well as a diet of 3.0 mm diameter moist pellet. Manufacture of the moist pellet is described

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in Chapter 1. Consequently a diet of flake food, supplemented by moist pellet, was used in all trials. Flake food was fed ad libitum at least once daily, usually in conjunction with smaller amounts of moist pellet.

Fish in three of the four indoor experiments were injected with low doses of a hormone (10 μg of luteinizing hormone releasing hormone analogue, des-Gly^{10}-[DAla^6], ethylamide - LHRHa) and a dopamine blocker (1 mg of pimozide) to encourage development of gametes and spawning behavior. In two trials fish were injected only on day 1 and in another trial they were injected on days 1, 3, 5, and 7. The indoor trials lasted from 12 to 30 d, and the outdoor trials lasted from 57 to 60 d.

The ratio of males to females varied among trials, and ranged from 2:1 to 1:5. Spawning success was evaluated by periodically examining spawning substrates and tanks for eggs or larvae.

Induced-Spawning Experiments

Fish captured from the wild and tame fish were given hormonal injections to induce ovulation or spawning in captivity. Wild fish were generally treated for disease prior to the first injection. Fish were considered tame (acclimated to captivity) if they had been held in captivity for several weeks, were feeding vigorously, and it seemed they could be held long-term in captivity. All tame fish used in induced-spawning trials were maintained in the oval raceway described in Chapter 1

at a constant temperature (≈ 22 C) and constant photoperiod (16 h light). At the end of induced-spawning trials, injected fish were placed into a section of the oval raceway, and some females were used in subsequent spawning trials after they had developed new complements of large oocytes. Males from which milt could be stripped also were used in subsequent trials. Thus, some tame fish were used in more than one spawning trial.

A three tank (110 L aquaria) system was used in all induced-spawning trials. The aquaria drained into a large Living Stream that received a steady, small input of activated charcoal-filtered municipal water. Water from the Living Stream was recirculated to aquaria by a submersible pump. Water temperature was controlled with a Frigid Units water chiller and a Blue-M submersible heater.

The hormones used were carp pituitary extract (CPE), human chorionic gonadotropin (hCG), and LHRHa. A dopamine blocker, domperidone (dom), was injected concurrently with LHRHa (LHRHa + dom). The hormones CPE and hCG also were injected concurrently in most trials (hCG + CPE). A few fish were given only CPE or hCG injections. If combined treatments (LHRHa + dom or hCG + CPE) were used, the chemicals were injected separately in consecutive injections. In most trials, the same hormonal treatment was used in successive injections of the same fish, but occasionally a different treatment was used. For example, some fish that had not spawned after two or three injections of LHRHa + dom were given hCG + CPE in subsequent injections. These treatments have been lumped into a

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common category called 'other', to simplify the description and analyses of induced-spawning trials.

Males and females received similar hormonal dosages, except that males from which milt could readily be expressed were not injected. Two to seven females, and two to four males were used per trial. The most common ratios used in tests were four or five females to three or four males.

In all cases, the first injection (primer injection) was of a lower dose than subsequent injections. The first high dose injection was usually given on the same day as the primer injection, and subsequent high dose injections were given on consecutive days thereafter.

All hormones were dissolved in 0.1 mL of 0.6 % NaCl solution and injected with a 26 gauge hypodermic needle and tuberculin syringe. Injections were given intraperitoneally at the base of the pelvic fin, because back-flow of injected solutions commonly occurred when injections were given intramuscularly.

The injection dosage that can induce spawning of the whitetail shiner is not known. Consequently, I made no attempt to regulate dosage of hormones on a per kilogram basis for each fish. Instead, injection dosages were based on a 12.5 g fish, and all fish were given the same volume of hormonal solution (0.1 mL) regardless of size. Dosages for primer injections (based on a 12.5 g fish) were 20 μg/kg of LHRHa, 2 mg/kg of domperidone, 200 I.U./kg of hCG, and 5 mg/kg of CPE. High dosages were 200 μg/kg of LHRHa, 20 mg/kg of domperidone, 2000 I.U./kg of hCG,

and 20 mg/kg of CPE. Total lengths of females were measured and recorded, and a weight-length equation (ln weight (g) = -13.24 + 3.36 * ln total length (mm)), developed from 36 gravid female whitetail shiners used in maturation experiments, was used to estimate weights and subsequent dosages for injected fish. The smallest mean diameter of oocytes in the ovaries of females used to develop the length-weight equation was 0.9 mm, because the germinal vesicle in oocytes of this size and larger can be induced to migrate with hormonal injections.

Weights of females that spawned were compared with those that did not for each treatment (hCG + CPE or LHRHa + dom) with Student's t-test. This test indirectly tested for differences in the efficacy of different hormone dosages to induce spawning of the whitetail shiner, because dosage was directly proportional to weight of females. The range, mean, and standard deviation of hormonal dosages that produced spawns were determined for each treatment.

The length of each fish was used as a mark to differentiate among fish used in each spawning trial. If lengths among fish used in a spawning trial were similar, a fin was also clipped to permit identification of fish.

Fish were captured from spawning tanks with a small, fine-mesh, aquarium dip net, and all fish were anesthetized with MS-222 (10 to 25 mg/L) prior to sampling of oocytes or injection of hormones. Fish usually succumbed to the anesthetic within one minute. Samples of oocytes were taken with a catheter from every female at the time of the first injection. The catheter was a short section of 1.2 mm I.D. and

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1.7 mm O.D. intramedic tubing attached to a tuberculin syringe. Oocytes were extracted by gently inserting the tubing into an ovary via the ovipositor and then applying suction. I tried to extract at least six of the largest size class of oocytes from the ovary for each sample. Smaller samples were taken if that number could not be obtained in two or three attempts, to prevent excessive stress to females. Positions of germinal vesicles and diameters were determined for several oocytes of the largest size class of oocytes in each sample.

Oocyte samples were usually taken at the time of all but the second injection, and sometimes on the day following the last injection. The injection and egg sampling schedules served to divide induced-spawning experiments into five relatively distinct time periods. At Time 1, fish received a primer injection, and the first sample of oocytes was taken; at Time 2, fish received the first high dose hormonal injection; and at Times 3 to 5, fish received additional hormonal injections and additional samples of oocytes were taken. These time periods are used in subsequent portions of this chapter.

The germinal vesicle was visible after oocytes were cleared in a solution of ethanol (60 %), formalin (30 %), and glacial acetic acid (10 %). This solution was superior to several other methods tested to clear oocytes of the whitetail shiner (Stoeckel and Neves 1992). After oocytes had been cleared, they were viewed under a dissecting microscope at 20X. An ocular micrometer was used to measure the shortest distance from the germinal vesicle to the periphery of the oocyte. Oocytes

were repositioned if necessary to obtain the measurement. Representative subsamples were examined in larger samples of oocytes. For example, if a sample of 20 oocytes of the largest size class contained ten oocytes in which the germinal vesicle had broken down, and the other ten had a germinal vesicle near the periphery, then two or three oocytes in each group were used. Thus, in samples where the mean ratio of germinal vesicle position to oocyte diameter equaled zero, all or nearly all oocytes were mature. The diameters of oocytes were measured at the same time that germinal vesicle positions were determined.

Females were considered to have successfully ovulated if at least 20 ova were spawned in an aquarium or if 20 ova minimum could be stripped from the fish. Females were examined to see if they had ovulated at the time of each injection or at time of egg sampling, and at the end of each trial by gently squeezing their abdomen.

Based on the outcome of induced-spawning trials, females were placed into one of five categories. One category included fish that died, and the other four described the fate of the oocytes: oocytes unovulated and atretic, oocytes unovulated but not atretic, oocytes mature and tank-spawned, and oocytes mature and strip-spawned.

Mean times between successive injections, mean ratios of germinal vesicle position to oocyte diameter, and mean diameters of oocytes, for fish that spawned and those that did not, were statistically compared with a general linear model

(GLM) to statistically assess differences between the two groups. The main effect in each model compared spawning individuals and nonspawning individuals, and each variable (injection times, germinal vesicle ratios, and diameters) was blocked by time period, because they were known or expected to vary with time. If differences, independent of time period, were significant, t-tests were used to determine where statistically significant differences existed.

Characteristics of Spawned Ova

The effectiveness of dry-stripping versus wet-stripping of ova was compared. Dry-stripped ova were stripped directly into the bottom of an 90 mm diameter by 50 mm high glass dish by gently squeezing the abdomen of ovulated females. Sperm, obtained by gently squeezing the abdomen of running males or by macerating testes dissected from nonrunning males, was added to the ova, and water from the spawning tank was then added to activate the sperm.

Wet-stripped ova were stripped into 90 mm diameter by 50 mm high glass dishes filled with water from the spawning tank. Ova stripped from the females generally adhered to the abdomen of females, and consequently, the stripping process entailed gently squeezing several oocytes from the female and then washing the oocytes into the glass dish. This process was repeated until all strippable ova were removed from the female. Sperm, obtained by stripping running males or macerated testes, were then added to the stripped ova in the water-filled dish. Milt obtained by

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stripping generally adhered to the abdomen of males, and consequently, it was also removed by washing the abdomen of the male in the water-filled dish.

Glass dishes that contained strip-spawned ova were submerged in 38 L aquaria two to three minutes after the addition of sperm. Extraneous materials were removed by swirling the water in the dish and pouring off suspended debris. An elongate air stone was placed atop dishes to provide circulation of water and oxygenation of spawned ova. Strip-spawned ova were examined at least once daily. At each examination, nonviable ova and dead embryos were counted and dislodged from the glass dish with a dissecting needle. They were subsequently removed from the dish by swirling the water and decanting suspended debris.

Fertilization success was estimated one day after spawning occurred, by counting the total number of live embryos (live embryos are clear, whereas nonviable ova and dead embryos are opaque) and comparing it with the total number of ova spawned. The viability of spawned ova was compared between wild and tame fish. Yolk and chorion diameters also were determined for several clutches that had viable ova. Measurements of yolk and chorion diameters of water-hardened ova were made under a dissecting microscope at 20X. Hatching success was estimated by comparing the total number of larvae hatched with the total number of live embryos at the beginning of the hatching period.

Number of ova spawned per tank-spawning or strip-spawning was determined for several females to estimate clutch sizes for hormonally-injected whitetail shiners.

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Tank-spawned ova were enumerated only if the clutch could be attributed to a single female, and only if spawning was known to have recently occurred (within several hours), because preliminary experiments indicated that adults would sometimes eat spawned ova. Overripe, nonadhesive ova were particularly susceptible to predation by adults.

The hatching times of several batches of spawned eggs were monitored. The number of days required for the majority of embryos to hatch was multiplied by the mean temperature during the hatching period to calculate number of degree-days required to hatch whitetail shiner eggs.

Results

Tank-Spawning Experiments

No embryos or fry were recovered in any of the tank-spawning trials (Table 2.1). Aggression of males, particularly highly tuberculated, brightly colored males, toward other males and females frequently occurred, but spawning behavior following aggressive displays was never observed. Aggressive behavior included chasing fish (males and females), nipping fins, and ramming other fish broadside. Mortality of one or more males often occurred within a few days after intense sparring by aggressive males. Thirty-six percent of the males and 26 % of females died in all tank-spawning trials combined.

Tank-spawning occurred in fish held in the oval raceway for induced-spawning experiments. Fry were noted on two occasions, and embryos were observed on one occasion. Spawning behavior was observed on five separate occasions. In addition, five females with free-flowing (ovulated) oocytes were recovered from the oval raceway. More than 100 either nonadhesive or only mildly adhesive (i.e., atretic) ova were obtained from four of these females. Twenty-four ova were obtained from the fifth female, and 13 (54 %) of them were viable.

Table 2.1. Results of tank-spawning experiments with <u>Cyprinella galactura</u>.

Tank type (number of spawns)	Temperature (C)	Trial length (d)	Day of hormone[1] injection	Sex ratio	Number dead
Circular (0)	15-31	60	None	8♂: 48♀	17 of 56
Circular (0)	16-28	57	None	12♂: 24♀	19 of 36
Aquaria (0)	25	15	None	1♂: 3♀	16 of 32
Aquaria (0)	25-28	30	1	1 or 2♂: 1 to 4♀	11 of 42
Aquaria (0)	26-28	20	1	1♂: 5♀	0 of 18
Aquaria (0)	24-28	12	1,3,5,7	1 or 2♂: 2 to 3♀	1 of 33

[1]LHRHa (10 μg/kg) + pimozide (1 mg/kg)

Induced-Spawning Experiments

Temperatures ranged from 20 to 27 C during induced-spawning trials, but they generally were between 22 and 26 C (90 % of the time). The mean temperature was 23.6 ± C. Mean time to first injection of fish captured from the wild was 39.3 h, and ranged from 3.0 to 113.5 h.

One hundred and sixty-one female whitetail shiners were injected in induced-spawning trials. They ranged in length from 60 mm to 130 mm, with a mean length of 106 ± 11 mm. The equation to convert total length of female to weight is ln

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weight (g) = -13.24 + 3.36 * ln length (mm) (N = 36, R^2 = 0.92), and estimated weight of the average female used in induced-spawning trials was 11.3 g. The estimated range in weights was 2 to 24 g. Thus, the dosages of hormones given to an average female were slightly higher than those listed in the Materials and Methods section for a 12.5 g fish, and estimated dosages ranged from 54 % to 625 % of the values listed in the Materials and Methods section for a 12.5 g fish.

The mean weight of females that spawned following injections of hCG + CPE was 10.3 ± 3.4 g (N = 16), and for those that did not spawn it was 10.5 ± 4.9 g (N = 29, Figure 2.1). The mean estimated weight of females that spawned following injections with LHRHa + dom was 12.6 ± 4.0 g (N = 23), and for those that did not spawn it was 12.0 ± 4.0 g (N = 71, Figure 2.1). The greater mean size of females injected with the LHRHa + dom treatment is due to the use of more tame fish, because tame females used in induced spawning trials were larger than wild fish on the average. Statistical comparisons within treatments indicated that the weights of females that spawned and those of fish that did not spawn did not vary significantly for either the hCG + CPE treatment (p=0.58) or the LHRHa + dom treatment (p=0.88). Consequently, within the range of values tested in this study, all dosages of hormones used to induce spawning of the whitetail shiner were equally effective.

The dosages of hormones that induced spawning in the whitetail shiner ranged from 150 to 3157 I.U./kg of hCG and 3.8 to 35.9 mg/kg of CPE for the hCG + CPE treatment, and from 120 to 890 μg/kg of LHRHa and 12.0 to 89.0 mg/kg of dom for
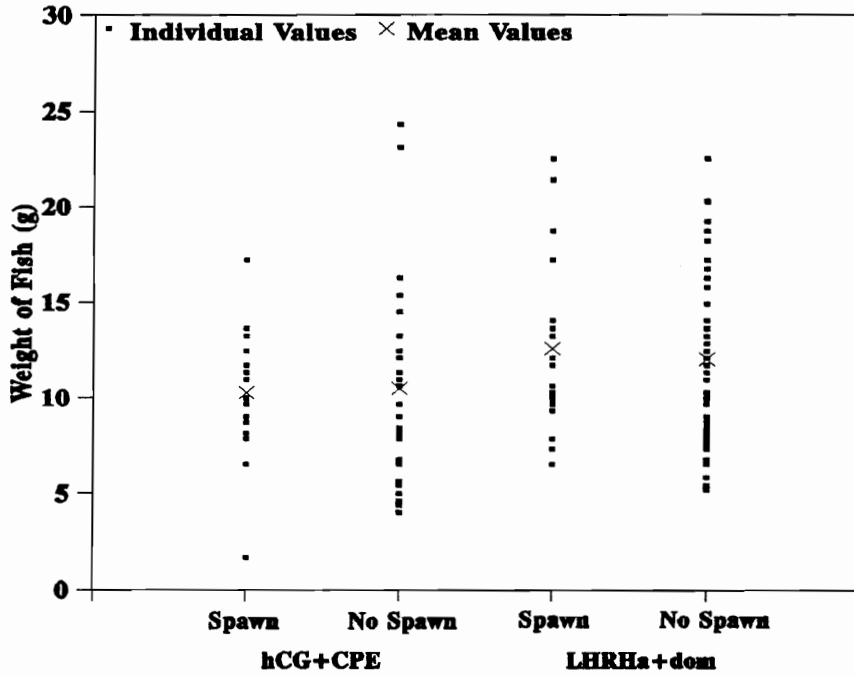
Figure 2.1. Weight of fish that spawned.

the LHRHa + dom treatment (Figure 2.2). The mean effective dosages were 1688 ± 811 I.U./kg of hCG and 19.5 ± 8.7 mg/kg of CPE for the hCG + CPE treatment, and 363 ± 200 μg/kg of LHRHa and 36.3 ± 20.0 mg/kg of dom for the LHRHa + dom treatment.

Most (74 %) of the hormonally-injected females did not ovulate (Table 2.2). The oocytes in several unovulated fish were obviously atretic at the time of the last egg sample, because the chorion had begun to deteriorate. However, this was difficult to determine in other fish, particularly those in which the germinal vesicle had broken down, whether oocytes were still viable or not. In addition, oocytes of hormonally-injected females that did not spawn commonly became atretic several

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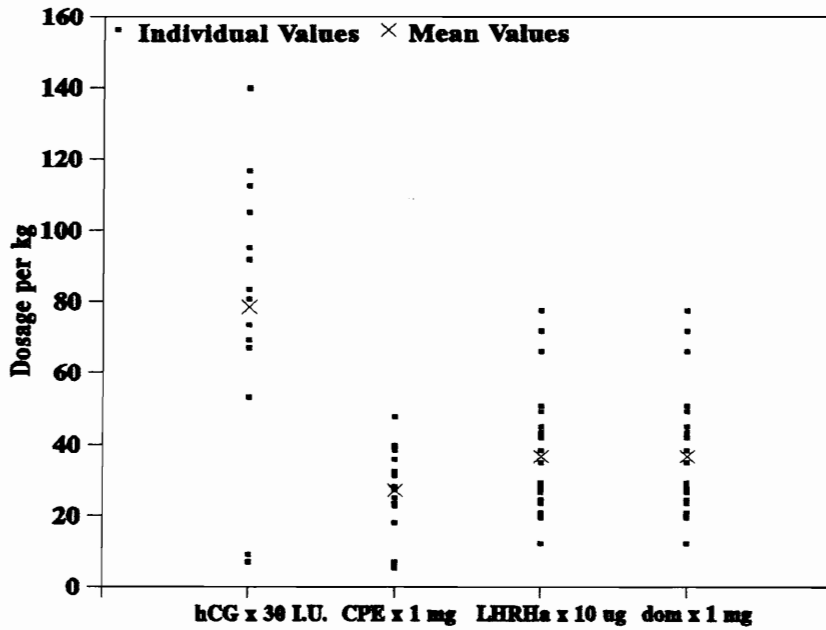
Figure 2.2. Dosages of hormones that induced spawning of female <u>Cyprinella</u> <u>galactura</u>.

Table 2.2. Results of strip-spawning experiments for <u>Cyprinella</u> <u>galactura</u>. Values are number of fish in each category.

Source of fish	Hormones injected	Results of trials				
		Female died	Oocytes unovulated	Oocytes atretic	Strip-spawn	Tank-spawn
Wild fish	hCG+CPE	0	16	4	6	5
	LHRHa+dom	2	23	0	2	1
	Other[1]	0	3	3	0	0
Domesticated fish	hCG+CPE	1	7	1	1	2
	LHRHa+dom	3	37	1	11	8
	hCG	0	6	0	0	0
	CPE	0	0	5	0	1
	Other	0	7	2	3	0

[1]Different hormonal treatments used in successive injections

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days after the end of a spawning trial. Consequently, the 'atretic' category was combined with the 'unovulated' category.

Slightly fewer fish tank-spawned ($N = 17$) than were strip-spawned ($N = 23$) following hormonal injections. Some fish were stripped after a few eggs had been tank-spawned. These two categories have also been combined to facilitate analysis of factors related to ovulation of hormonally-injected whitetail shiners. The first few fish that tank-spawned did not use the tile spawning substrates. Instead, they used the bottom edges and corners of the aquarium. The tile spawning substrates were subsequently removed to facilitate capture of fish for hormonal injections and egg sampling. All subsequent tank-spawning was also along the bottom edges and corners of aquaria.

Spawning behavior was observed on several occasions, and it varied substantially among groups of fish. In some, a single male would control the tank, and in others, two or more (sometimes all) males would participate in the spawning ritual. Subdominant males were relegated to the upper portion of the water column. Females typically were kept toward the top of the tank by aggressive males. The ritual normally consisted of a male (or males) making passes along the long edge of the tank bottom. The frequency and intensity of these passes varied considerably among males. A shuddering of the body (presumed to be associated with release of sperm) often accompanied the passes. In the more intense behavior, males circled in a vertical plane numerous times; the bottom of the arc consisted of a pass along

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the bottom edge of the tank. Females would sometimes follow the male as he made passes, and she also would shudder when passing the bottom edge of the tank (presumably a prelude to expulsion of ova). Occasionally males would accompany females during the passes. His position was normally just dorsal and to the side of the female in these instances. Very aggressive males would sometimes 'cut out' a female from the group and encourage her to accompany him along passes by breaking from a circular pass cycle and swimming near, sometimes chasing, the female. At other times the female took the initiative to begin the pass cycle. In several instances females that were 'cut out' from the others spawned. But neither this behavior, nor that of females that initiated spawning behavior, were indicative of imminent spawning.

Only six females died; four of these either jumped from the tank or died from an overdose of anesthetic. These fish were excluded from further analyses. Several males also died or were killed during induced-spawning experiments. Individuals were sacrificed to fertilize strip-spawned ova; others jumped from the spawning tank, died from an overdose of anesthetic or apparently as a result of handling and spawning-induced stress. Incomplete records on the ultimate fate of all males precluded placement of dead males into categories, but the number of deaths that could be attributed to handling and spawning-induced stress was low. Brightly colored males, from which milt could not be expressed at the beginning of a spawning trial, nearly always produced milt when stripped after one or two hormonal injections.

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Table 2.3. Comparison of efficacy of hormonal treatments to induce oocyte maturation (germinal vesicle (GV) breakdown) and spawning of <u>Cyprinella</u> <u>galactura</u>. Values are percent of fish in each category.

Source of fish	Hormones injected	Number of fish	Hours to GV breakdown				Percent that spawned
			<12[1]	12-30	>30	None	
Wild fish	hCG+CPE	31	10	58	6	29	35
	LHRHa+dom	28	18	25	7	50	11
	Other[2]	6	0	0	83	17	0
Domes-ticated fish	hCG+CPE	14	14	50	29	7	21
	LHRHa+dom	58	3	24	21	52	33
	hCG	6	0	0	17	83	0
	CPE	6	0	100	0	0	17
	Other	12	0	33	33	33	25
Total	All	161	7	34	19	40	25

[1]Oocytes of all fish were mature at time of first sample (time = 0 h)
[2]Different hormonal treatments used in successive injections

The hCG + CPE and LHRHa + dom treatments induced spawning of whitetail shiners. The hCG + CPE treatment was more effective in inducing spawning of wild fish, whereas the LHRHa + dom treatment was more effective for tame fish (Table 2.3). Overall, the two treatments were comparable, as 24 % of LHRHa + dom and 31 % of hCG + CPE injected fish spawned. All treatments, including those classified as 'other', were combined to facilitate identification of factors associated with ovulation of hormonally-injected whitetail shiner females.

Table 2.4. Time (h) of successive hormonal injections and egg samples, relative to first time period.

Time period	Injection times		Egg sampling times	
	Mean ± SD[1] (number)	Range	Mean ± SD (number)	Range
1	0 (155)	NA[2]	0 (155)	NA
2	7.2 ± 5.9 (155)	1-26	NA (0)	NA
3	24.8 ± 6.4 (117)	14.0-43.5	25.4 ± 7.0 (133)	14.0-43.5
4	43.7 ± 9.5 (27)	25.0-51.5	51.3 ± 15.1 (74)	25.0-89.5
5	59.5 ± 11.3 (9)	52.0-74.5	75.9 ± 40.1 (23)	43.0-167.5

[1]One standard deviation
[2]Not applicable

Three hundred eighty-five oocyte samples were collected over five time periods (Table 2.4). The mean number of oocytes examined per sample was 4.7 ± 0.9. Information on oocytes and injection times was analyzed to identify criteria for development of injection protocols and selection of female Cyprinella spp. for induced spawning. One hundred fifty-three females were given at least two injections; 117 were injected three times, 27 four times, and 9 were injected five times. The range of times within which fish were injected in each time period was relatively wide (Table 2.4), but most injections were concentrated toward the mean (Figure 2.3). Oocytes were sampled from all fish at Time 1, and from 133, 74, and
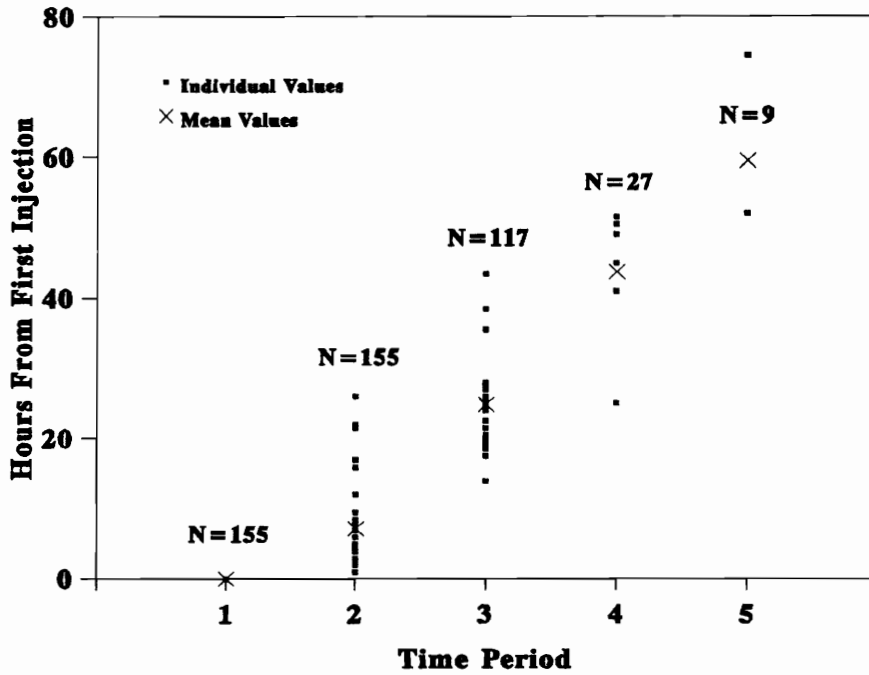
Figure 2.3. Time of hormonal injections in each time period for all spawning trials. 23 fish at Times 3 to 5.

The overall statistical probability that injection times did not vary among spawners and nonspawners was 0.11, and this value was not considered to be significant. Consequently, post-hoc comparisons were not run. However, because most oocytes matured and most spawning occurred after two or three injections (Table 2.3 and Figure 2.4), it is worth noting that the mean number of hours at Time 2 and Time 3 was less for fish that spawned (Table 2.5).

At Times 1, 3, 4, and 5, the mean ratios of position of germinal vesicle to diameter of oocytes were 0.28, 0.15, 0.16, and 0.10 for nonspawners and 0.28, 0.04, 0.02, and 0.00 for spawners, respectively (Table 2.6). The overall p-value for

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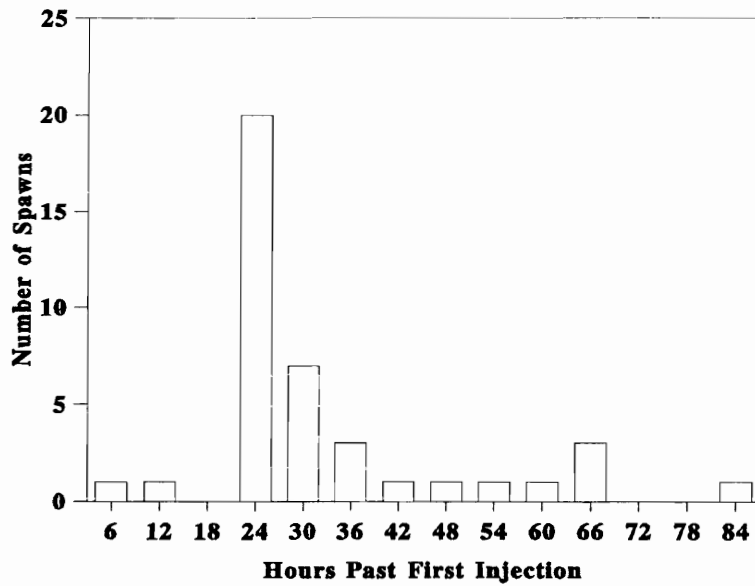
Figure 2.4. Time to spawning of hormonally-injected female <u>Cyprinella</u> <u>galactura</u>, relative to first injection.

Table 2.5. Comparison of time (h) of hormonal injections, between fish that ovulated (i.e., fish that tank-spawned or were strip-spawned) and those that did not.

Time period	Spawners		Nonspawners	
	Mean ± SD[1] (N)	Range	Mean ± SD (N)	Range
1	0 (40)	NA[2]	0 (115)	NA
2	5.7 ± 3.5 (40)	1.0-17.0	7.7 ± 6.5 (115)	1.0-26.0
3	22.5 ± 5.8 (14)	14.0-35.5	25.1 ± 6.5 (103)	14.0-43.5
4	46.4 ± 4.3 (4)	41.0-50.5	43.3 ± 10.2 (23)	25.0-51.5
5	NA (0)	NA	59.5 ± 11.3 (9)	52.0-74.5

[1]One standard deviation
[2]Not applicable

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Table 2.6. Comparison of ratios of germinal vesicle position to diameter of oocytes between hormonally-injected <u>Cyprinella</u> <u>galactura</u> that ovulated (i.e., fish that tank-spawned or were strip-spawned) and those that did not.

Time period	Spawners		p value	Nonspawners	
	Mean ± SD[1] (N)	Range		Mean ± SD (N)	Range
1	0.28 ± 0.11 (40)	0.0-0.44	0.792	0.28 ± 0.13 (115)	0.0-0.46
3	0.04 ± 0.08 (40)	0.0-0.35	0.003	0.15 ± 0.14 (109)	0.0-0.42
4	0.02 ± 0.09 (13)	0.0-0.44	0.212	0.16 ± 0.12 (68)	0.0-0.36
5	0.0 (2)	NA[2]	0.439	0.10 ± 0.12 (22)	0.0-0.39

[1]One standard deviation
[2]Not applicable

differences among ratios of germinal vesicle position to diameter of oocytes was

< 0.001, and a statistically significant difference occurred at Time 3 ($\underline{p} = 0.003$).

The ratio of germinal vesicle position to oocyte diameter was zero (i.e.,

oocytes were mature or matured) in 97 of 155 female whitetail shiners used in

induced-spawning trials. Oocytes matured in 85 fish after they were given hormonal

injections. If oocytes responded to hormonal injection they generally did so fairly

quickly, as indicated by the proportions of fish in which the germinal vesicle broke

down before 30 h, and after 30 h (Table 2.3), and the time at which most spawning

occurred (Figure 2.4). Oocytes of 62 fish matured by Time 3 (73 %), 14 matured by

Time 4 (16 %), and 9 matured by Time 5 (11 %). The mean diameter of oocytes that matured was 1.08 mm at Time 1, and 1.18 mm after maturation occurred. Thus, they increased an average of 0.1 mm in diameter. The diameter of oocytes at time of maturation (range = 0.88 to 1.75 mm) was not correlated with total length of fish (range = 60 to 130 mm; R^2 = 0.01). The initial mean ratio of germinal vesicle position to oocyte diameter was 0.30 ± 0.10 for fish in which oocytes matured after hormonal injections, and ranged for 0.06 to 0.46. Twelve fish had mature oocytes at Time 1, but only three of these spawned following hormonal injections.

Maturation of oocytes in eight of the 40 fish that spawned was observed several hours before ovulation occurred. Time to spawn from the observed time of germinal vesicle breakdown ranged from 3 to 41 h and averaged 20.2 ± 12.0 h.

The overall p-value for differences among diameters of oocytes was also significant ($p < 0.001$). The mean diameters of oocytes of fish that spawned were greater than those that did not spawn at all time periods (Table 2.7). Differences between oocyte diameters were statistically significant at Time 1 and Time 2 ($p < 0.01$). The smallest initial mean size of oocytes of a female that spawned was 0.96 mm.

Characteristics of Spawned Ova

Viable ova of strip-spawned whitetail shiners were very adhesive. Most dry-stripped ova were lost prior to fertilization because they were difficult to remove

Table 2.7. Comparison of diameters of oocytes from hormonally-injected <u>Cyprinella</u> <u>galactura</u> that ovulated (i.e., fish that tank-spawned or were strip-spawned) and those that did not.

Time period	Spawners		p value	Nonspawners	
	Mean ± SD[1] (N)	Range		Mean ± SD (N)	Range
1	1.12 ± 0.13 (40)	0.96-1.66	0.008	1.06 ± 0.12 (115)	0.84-1.50
3	1.18 ± 0.10 (24)	1.04-1.41	0.000	1.08 ± 0.11 (109)	0.81-1.43
4	1.26 ± 0.19 (8)	0.91-1.55	0.138	1.15 ± 0.13 (68)	0.90-1.65
5	1.22 (1)	NA[2]	0.627	0.1.16 ± 0.12 (22)	1.00-1.42

[1]One standard deviation
[2]Not applicable

from the abdomen of the female, and adhered to sides of the spawning dish and fingers. Ova could be washed from the abdomen of wet-stripped females, and the washing action dispersed ova relatively uniformly on the bottom of the dish. Atretic ova were not adhesive.

Fertilization success was estimated for 34 clutches of ova (Table 2.8), and it ranged from 0 % to 85 %. More than 50 % of ova were estimated to be viable in only one clutch of wild fish (9 %), compared to 15 clutches of tame fish (65 %). Mean yolk diameters of water hardened ova for three clutches with viable ova were 1.01 mm, 1.06 mm, and 1.36 mm, and the corresponding chorion diameters were

Table 2.8. Fertilization success of wild and tame Cyprinella galactura following hormonal injection.

Source of fish	Fertilization success[1]	Number of fish	Hours to spawn (mean)	Mean number of injections
Wild	Low	6	15-46 (27)	1.9
fish	Medium	4	10 (10)	1.0
	High	1	25 (25)	2.0
Domesticated	Low	5	41-119 (72)	3.6
fish	Medium	3	18-21 (20)	2.0
	High	15	8-35 (23)	2.1

[1]Low < 20 %, Medium = 20 to 50 %, and High > 50 %

1.74 mm, 1.77 mm, and 2.01 mm. Percent hatch of five strip-spawned clutches ranged from 30 % to 67 %, and averaged 52 %.

Clutch sizes were estimated for 26 clutches produced by hormonally-injected whitetail shiner females, and they ranged from 20 to 1650 ova (Figure 2.5). The median value was 400 ova. Eleven of 15 tank-spawnings occurred before 12:30 h, and four occurred after 15:30 h. Approximately equal numbers of fish (12 and 13) were strip-spawned during the same time periods.

Time to hatch was determined for seven clutches. The first cleavage occurred in less than two hours at 23 C, and embryos were noticeable within 24 h. Hatching was spread out over several days (usually three to four), but the majority of larvae hatched within the same day. Ova hatched in 10 d at 23 C, 8 d at 24 C, 8 d at 25 C
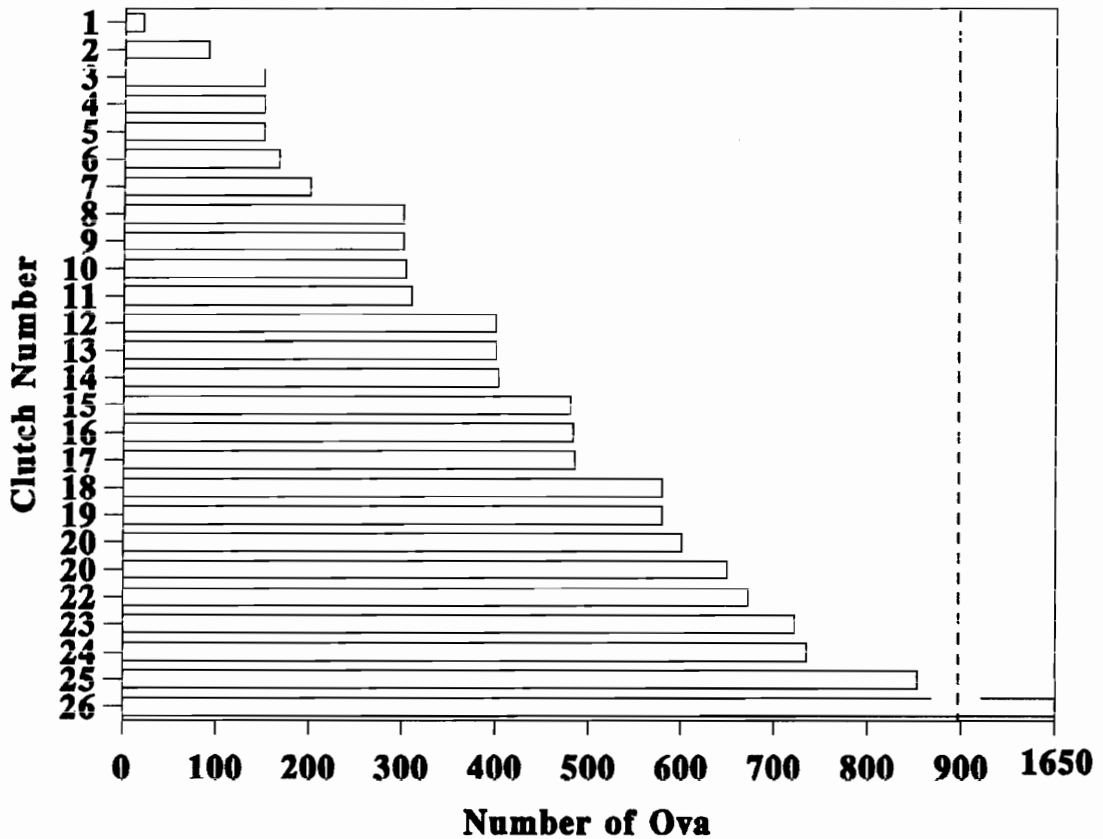
Figure 2.5. Size of clutches produced by hormonally-injected female <u>Cyprinella</u> <u>galactura</u>.

(three clutches), and 7 or 8 d at 26 C. The number of degree(C)-days ranged from 182 to 230, with a mean of 201 degree(C)-days. Larvae without inflated swim bladders were noticed in some fish toward the end of this study. Some clutches had nearly 0% inflation of swim bladders.

Discussion

Tank-Spawning Experiments

Mortality of fish was high in four of the six trials, and attributed primarily to aggressiveness of males. In aquaria with only one male, aggression of the male toward females, although less intense than that toward other males, was common. In indoor trials in which substantial mortality occurred, nearly all males were highly tuberculated and brightly colored, whereas in the other two indoor trials, secondary sexual characteristics of males were less well developed. Brightly colored males were more skittish and typically became very agitated when approached. Their alarm behavior catalyzed a heightened nervous response of other fish in the tank. Most fish fed well throughout the trials, but very aggressive males sometimes were too unsettled to feed well. Aggression of males seemed to vary in direct proportion to spawning readiness. Thus, mortality of fish is most likely to occur when they are in prime reproductive condition, and the potential for spawning the whitetail shiner in aquaria seems to be low.

Mortality of whitetail shiners in the larger tanks is more difficult to explain, because I maintained fish in reproductive condition without undue difficulty in sections of the oval raceway (see Chapter 1). Number of mortalities increased as the season progressed, and consequently it was correlated with an increase in number of breeding males and temperature. Healthy fish fed well throughout the trials.

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Although fish were treated periodically for disease, Columnaris was noted on several occasions. I suspect that mortalities associated with secondary Columnaris infections were fostered by the mechanical damage inflicted by aggressive males in the outdoor tanks, particularly at higher temperatures. The lack of verifiable reproduction in either of the two outdoor tanks over a 60 d period indicates that the potential for propagation of the whitetail shiner using these large circular tanks, under the conditions employed in this study, is low. Reduction of density of fish or temperature may enhance the potential for this system.

Several tank-spawnings were observed in the oval raceway. However, the verification of only three clutches of spawned ova in two years of holding fish in the raceway and the occurrence of atretic ova in four of five females with free-flowing, ovulated ova, indicates that this system is also unsatisfactory for tank-spawning whitetail shiners for controlled propagation efforts.

The ability to adapt to captivity apparently varies among species of Cyprinella. For example, some species such as the steelcolor shiner, Cyprinella whipplei, and the blacktail shiner, Cyprinella venusta, readily spawn on the same day, or within a couple days of capture (Pflieger 1965). The whitetail shiner does not. Mark Ferguson (personal communication, Virginia Department of Game and Inland Fisheries) held five species of Cyprinella in the laboratory under identical conditions. Two of them readily spawned in captivity, one spawned infrequently, and two spawned very infrequently or not at all (Table 2.9). A rating of easy indicates the

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Table 2.9. Ratings of ability to spawn species of Cyprinella in captivity, and legal status of rare Cyprinella spp.

Species of Cyprinella	Rating of ability to spawn in captivity	Reference
analostana[1]	Easy	Gale and Buynak 1978
callistia[1]	Difficult	Ferguson 1990
cloristia	Difficult	Ferguson P.C.[5]
galactura	Difficult	this study
gibbsi	Easy	Ferguson P.C.
leedsi	Easy	Heins & Rabito 1986
lutrensis[1]	Easy	Hubbs & Strawn 1956, Gale 1986
pyrrhomelas	Medium	Ferguson P.C.
spiloptera[1]	Easy	Pflieger 1965, Gale & Gale 1977
trichroistia	Easy	Ferguson P.C.
venusta[1]	Easy	Hubbs & Strawn 1956, Heins 1990
whipplei[1]	Easy	Pflieger 1965
caerulea[2]		
callitaenia[2,3]		
camura[1]	Other rare Cyprinella spp.[6]	
formosa[4]		
monacha[3,4]		
proserpina[2,3]		

[1]Special Concern, Statewide
[2]Special Concern, Federally
[3]Protected, Statewide
[4]Protected, Federally
[5]Personal communication
[6]Little or no information on ability to spawn these species

species apparently readily spawns in captivity, whereas a rating of difficult indicates that use of hormonal injections is probably necessary to produce an adequate supply of fertilized ova for captive propagation.

Induced-Spawning Experiments

Few mortalities of fish used in induced spawning experiments occurred as a result of handling. Consequently, it seems that the handling methods employed in this study are satisfactory for use with other Cyprinella. Temperature was ≈ 22 C in the holding tank, and generally < 25 C in the spawning tanks. Several fish jumped from the spawning tank when attempts were made to net them for additional injections. Use of a larger net (approximately tank width) permitted capture of all fish in the tank with one pass of the net and deterred jumping. The larger net also reduced the amount of time, and subsequent stress, required to chase and capture all fish. Doses of 10 to 25 mg/l of MS-222 are adequate for anesthetizing Cyprinella spp. However, mortality of whitetail shiners can result if they remain in the solution too long. This occurred on a couple of occasions when distractions, such as strip-spawning fish, prevented a prompt return of fish to the spawning tank. Consequently, a person should be assigned to monitor anesthetized fish when working with rare Cyprinella spp.

The hCG + CPE treatment seemingly is a more powerful inducer of final maturation of oocytes Cyprinella spp. than the LHRHa + dom treatment, because

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≥ 50 % of injected females matured in less than 30 hours following hCG + CPE injections, compared to ≤ 25 % of those injected with LHRHa + dom at the dosages used in this study. CPE seems to be the primary factor inducing maturation, as oocytes in only one of six females injected with hCG matured compared to six of six for CPE-injected females. Maturation of oocytes and spawning occurred ≤ 36 h in most fish that responded to hormonal injections. Thus, if Cyprinella spp. females respond to the injection protocols used in this study, they will generally do so within 36 h (i.e., after two or three injections).

A problem encountered in this study was the lack of ovulation in many females with mature oocytes; only 41 % of females with mature ova spawned. Instead, many mature ova became atretic during or shortly after trials were terminated. The slightly shorter times between the first and third injections of fish that spawned compared to those that did not spawn infers that a shorter time between injections will increase the percentage of fish that spawn.

The dosages of hormones and dopamine blockers given to fish in induced-spawning experiments are on the high end of those reported in the literature for spawning other species (Table 2.10), but the range of effective doses varies widely among species (Lam 1982, Zohar 1986, Peter et al. 1988). The possibility exists that high doses of hormones overwhelmed the ovarian ovulation mechanism of some fish in this study. CPE doses, in particular, seem to be higher than those required, because oocytes in all females (N=6) injected with CPE were atretic within 30 h.

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Table 2.10. Examples of dosages, time between injections, and time to spawn for hormones used to spawn cyprinids.

Species	First Injection[1]	Second Injection (Time in h)[2]	Third Injection (Time in h)	Hours to Ovulation[3]	Source
Cyprinus carpio	2-4 mg/kg CPE	---	---	12-14	Peter et al. 1988
	800-1000 I.U./kg hCG	---	---	12-14	Peter et al. 1988
	5 mg/kg dom + 10 ug/kg LHRHa	---	---	14-16	Peter et al. 1988
	1 mg/kg dom + 10 ug/kg sGnRHa	---	---	14-16	Peter et al. 1988
	5.5 mg/kg CPE + 27.5 mg/kg chloramphenicol	---	---	24	Sorenson 1971
	0.6 mg/kg CPE	2 mg/kg 17α-20β-hydroxy-dihydroprogesterone (24)	---	24	Jalabert et al. 1977
Carassius auratus	3000 I.U./kg hCG	---	---	24-48	Yamamoto et al. 1966
	4.4-6.6 mg/kg CPE	---	---	12-20	Clemens and Sneed 1962
	100-1600 I.U./fish hCG	---	---	20-22	Sneed and Clemens 1959
	10 mg/kg pim + 100 ug/kg LHRHa	100 ug/kg LHRHa (3)	---	< 23	Sokolowska et al. 1984
	10 mg/kg pim	100 ug/kg LHRHa (3)	---	< 23	Sokolowska et al. 1984
Hypopthalmichthys molitrix	100-200 I.U./kg hCG	400 I.U./kg hCG + 10 ug/kg LHRHa (5-6)	---	6-8	Peter et al. 1988
	2-4 mg/kg CPE	10-20 mg/kg CPE (5-6)	---	6-8	Peter et al. 1988
	100-200 I.U./kg hCG	700-1000 I.U./kg hCG (5-6)	---	6-8	Peter et al. 1988
	5 mg/kg dom + 50 ug/kg LHRHa	---	---	8-12	Peter et al. 1988

Table 2.10 continued. Examples of dosages, time between injections, and time to spawn for hormones used to spawn cyprinids.

Species	First Injection[1]	Second Injection (Time in h)[2]	Third Injection (Time in h)	Hours to Ovulation[3]	Source
	5 mg/kg dom+ 10 ug/kg sGnRHa	---	---	8-12	Peter et al. 1988
	5 mg/L CPE	10 mg/L CPE (6)	---	---	Saidin et al. 1988
	50 I.U./kg hCG	250 I.U./kg hCG (24)	4 mg/kg CPE (6)	---	Saidin et al. 1988
	5-10 ug/kg LHRHa	15 ug/kg LHRHa (6-20)	---	4-21	Ngamvongchon et al. 1988
	200 I.U./kg hCG	1500-1800 I.U./kg hCG (6)	---	6	Ngamvongchon et al. 1988
Ctenopharyngodon idella	1 mg/kg CPE	4-10 mg/kg CPE+ 10 ug/kg LHRHa (5-6)	---	6-8	Peter et al. 1988
	1-2 mg/kg CPE	6-12 mg/kg CPE (5-6)	---	6-8	Peter et al. 1988
	10 ug/fish LHRHa	2-4 mg/kg CPE+ 10 ug/kg LHRHa (5-6)	---	6-8	Peter et al. 1988
	5 mg/kg dom+ 10 ug/kg LHRHa	---	---	8-12	Peter et al. 1988
	50 I.U./kg hCG	1 mg/kg CPE (24)	4 mg/kg CPE (6)	---	Saidin et al. 1988
	50 I.U./kg hCG	250 I.U./kg hCG (24)	4 mg/kg CPE (6)	---	Saidin et al. 1988
	50 I.U./kg hCG	250 I.U./kg hCG+ 1 mg/kg CPE (24)	6 mg/kg CPE (6)	---	Saidin et al. 1988
	50 I.U./kg hCG	250 I.U./kg hCG+ 1 mg/kg CPE (24)	6 mg/kg CPE (6)	---	Saidin et al. 1988
Aristichthys nobilis	100-200 I.U./kg hCG	400 I.U./kg hCG+ 10 ug/kg LHRHa (6-8)	---	6-8	Peter et al. 1988
	2-4 mg/L CPE	10-20 mg/L CPE (6-8)	---	6-8	Peter et al. 1988

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Table 2.10 continued. Examples of dosages, time between injections, and time to spawn for hormones used to spawn cyprinids.

Species	First Injection[1]	Second Injection (Time in h)[2]	Third Injection (Time in h)	Hours to Ovulation[3]	Source
	100-200 I.U./kg hCG	700-1000 I.U./kg hCG (6-8)	---	6-8	Peter et al. 1988
	5 mg/kg dom+ 50 ug/kg LHRHa	---	---	8-12	Peter et al. 1988
	250 I.U./kg hCG	4 mg/L CPE (6)	---	---	Saidin et al. 1988
	50 I.U./kg hCG	250 I.U./kg hCG (24)	4 mg/kg CPE (6)	---	Saidin et al. 1988
	5-10 ug/kg LHRHa	15-20 ug/kg LHRHa (6-20)	---	4-24	Ngamvongchon et al. 1988
	200 I.U./kg hCG	1500-1800 I.U./kg hCG (6)	---	6	Ngamvongchon et al. 1988
Mylopharyngodon piceus	2 mg/L CPE+ 100 ug/kg LHRHa	10-16 mg/L CPE+ 100 ug/kg LHRHa (4-5)	---	8-10	Peter et al. 1988
	2-4 mg/L CPE	16-30 mg/L CPE (6-8)	---	8-10	Peter et al. 1988
	10 mg/kg pim+ 100 ug/kg LHRHa	5 mg/kg pim+ 50 ug/kg LHRHa (6)	---	6-8	Peter et al. 1988
Semotilus atramaculatus	0.25 CP/fish	---	---	48	Ball and Bacon 1954
Notemigonus chrysoleucas	1-2 mg/fish CPE	---	---	15	Clemens and Sneed 1962
Notropis umbratilis	1-2 mg/fish BPE	---	---	10-20	Clemens and Sneed 1962
Notropis atherinoides	1-2 mg/fish BPE	---	---	20-24	Clemens and Sneed 1962

[1]CPE=carp pituitary extract, BPE=buffalo (Ictiobus spp.) pituitary extract, hCG=human chorionic gonadotropin, LHRHa=luteinizing hormone releasing hormone analogue, sGnRHa=salmon gonadotropin releasing hormone analogue, pim=pimozide, dom=domperidone
[2]time past previous injection
[3]time from last injection to ovulation

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Spontaneous ovulation in the goldfish, Carassius auratus, is significantly influenced by temperature, photoperiod, and the presence of vegetation (Stacey et al. 1979). The temperature and photoperiod regime used in this study is probably adequate for spawning Cyprinella spp. However, modification of the spawning tanks may increase ovulation by providing conditions more amenable to spawning. Use of larger tanks, or the inclusion of spawning substrates of a larger size or different type than those used in preliminary trials, may be beneficial.

The effects of ovarian resorption of atretic oocytes on production of new clutches of oocytes in Cyprinella spp. are not known. Bieniarz and Epler (1976) found that resorption of atretic oocytes in the common carp, Cyprinus carpio, following hormonal injection can be a very slow process, and that the stage of maturity of oocytes not resorbed remains unchanged. In female whitetail shiners that had atretic ova following hormonal injections, little extraneous material from resorbed oocytes remained, and larger oocytes seemed to be competent after approximately four weeks (see Chapter 1).

Selection of females for hormonal injections was not facilitated by position of the germinal vesicle. As expected, lower ratios of germinal vesicle position to diameter of oocyte were observed for fish that spawned compared to those that did not at Times 3 to 5, due to the migration and breakdown of the germinal vesicle in fish that spawned. Small sample sizes precluded the detection of statistically significant differences at Time 3 and Time 4. I expected that the mean ratios at

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Time 1 would provide useful information to help differentiate potential spawners from nonspawners, but the ratios for both groups were exactly the same (0.28). Thus, the ratio of germinal vesicle position to diameter of oocyte does not help to differentiate between prospective females of Cyprinella spp. that are likely to spawn, and those that are not.

Diameter of oocytes seems to be a useful indicator of competency of oocytes to respond to hormonal injections. The smallest mean size of the largest size class of oocytes at Time 1 that was spawned following hormonal injections was 0.96 mm. The smallest size of oocyte that responded to hormonal therapy (i.e., germinal vesicle breakdown occurred) was 0.90 mm (7 of the 30 fish). Thus, the smallest size of oocyte competent to respond to hormonal therapy was approximately 0.1 mm smaller than the smallest mean size of oocyte that was ovulated following hormonal injections. This type of relationship can probably be used to establish minimum oocyte diameter criteria for selection of other Cyprinella spp. females for spawning. Mean diameter of the largest oocyte size class to be used for induced spawning of whitetail shiners should be at least 1.0 mm.

Conditions for Successfully Spawning Fish

Very few larvae were produced using the dry-stripping method because many ova were damaged or lost during the stripping process due to their extreme adhesiveness. Strawn and Hubbs (1956) reported similar problems with stripped ova

of red shiner, Cyprinella lutrensis. They used pond snails to clear up dead eggs and larvae, and removed clumps of fungused embryos with a pipette.

Wet-stripping proved satisfactory for strip-spawning Cyprinella spp. The best procedure was to gently strip a few ova from the female over a dish. The ova typically would adhere to the abdomen of the female but could be dislodged by washing her abdomen in the dish. The washing action helped to disperse and prevent clumping of ova. Clumping of ova should be avoided because the clumps are very susceptible to Saprolegnia infections, which subsequently kill the embryos. Several dishes of the size used in this study, or larger dishes, should be used for large clutches of spawned ova to preclude crowding and subsequent clumping of ova.

Milt should be stripped from males as soon as possible after ova are stripped from females. The volume of milt is small, and it generally adheres to the abdomen of the male. It can be washed into the dish using the same technique for stripping gametes from the female. Selection of males for milt-production was not a problem in spawning trials, if highly tuberculated brightly colored males were used. The use of several males and hormonal injections is encouraged to help ensure availability of sperm at the time of strip-spawning. If only one male is used in the spawning tank with females, others can be held in alternate tanks. They should also be injected with hormones.

Males and females should be anesthetized prior to strip-spawning or injections because adult Cyprinella spp. are small and hyperactive. Significant mechanical damage (e.g., scale loss and mucous removal) can result if fish are not anesthetized.

A notable difference in fertilization success occurred between wild and tame fish. This difference is attributed to stress incurred by wild fish during the capture and handling process, and a subsequent decline in the capacity or competency of oocytes to respond to hormonal injections. The average time to first injection of wild fish following capture of wild females was 39 ± 33 h (range = 3 to 114 h, N = 65). Hormonal injection immediately after capture may preclude a potential reduction in competency of oocytes. However, the ability to maintain Cyprinella spp. indefinitely in reproductive condition in captivity (see Chapter 1) negates the need to inject wild fish.

Wild fish should be acclimated to captive conditions before hormonal injections are given to induce spawning. Acclimation of wild fish to captivity precludes the need to select individuals in prime reproductive condition in the field for hormonal injection, because Cyprinella spp. can be brought into spawning condition in captivity (see Chapter 1). The ability to use fish in less than prime reproductive condition significantly enlarges the size of the pool from which potential candidates for propagation can be selected; a principal consideration for rare Cyprinella spp. Another reason for not injecting wild fish in prime reproductive condition is that they, particularly males, are more susceptible to mortality induced

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by capture and handling stress. Acclimation of wild Cyprinella spp. would also permit time to determine whether a particular species will readily produce a satisfactory number of fertilized ova by tank-spawning in captivity, or whether use of hormonal injections will be required.

The median clutch size of spawned ova for hormonally-injected whitetail shiners was 400 ova. Reported clutch sizes for other species Cyprinella are 156 to 986 for the spotfin shiner, Cyprinella spiloptera (Gale and Gale 1977), 6 to 634 ova for the satinfin shiner, Cyprinella analostana (Gale and Buynak 1978), 26 to 228 ova for the bannerfin shiner (Rabito and Heins 1985), and 131 to 1661 ova for the red shiner (Gale 1986). Sizes of clutches of mature oocytes were 404 to 1815 for the whitetail shiner (Outten 1958), 88 to 230 for the bluestripe shiner, Cyprinella callitaenia (Wallace and Ramsey 1981), 150 to 800 for the spotfin chub, Cyprinella monacha (Jenkins and Burkhead 1984), 140 to 457 for the blacktail shiner (Heins and Baker 1987), and 44 to 570 for the Alabama shiner, Cyprinella callistia (Ferguson 1990). The clutch sizes of mature oocytes for whitetail shiners reported by Outten (1958) were larger than the clutch sizes of spawned ova he observed in the wild. Information is not available on whether naturally spawned clutches of other Cyprinella spp. are smaller than clutch sizes of mature oocytes. Clutch sizes of hormonally-injected female whitetail shiners in this study are comparable to the clutch sizes of mature oocytes reported by Outten (1958) for the species. This implies that most mature oocytes are induced to ovulate by hormonal injections, thereby creating

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larger clutch sizes of spawned ova than those produced by natural spawning. All but two clutches of hormonally-injected whitetail shiner females contained ≥ 150 ova, and this size is considered satisfactory for propagating rare Cyprinella spp.

Most tank-spawnings of hormonally-injected whitetail shiners occurred in the morning, and this is similar to reports for other species of Cyprinella (Gale and Gale 1976, Gale and Buynak 1978, Gale 1986). Number of strip-spawnings was evenly distributed between morning and other times. Whether some fish that were strip-spawned would have tank-spawned the following morning is unknown. I did not determine the length of time that ovulated oocytes remain viable in the whitetail shiner, but times vary with species and temperature (Horvath 1986). It does not seem to be greater than 24 h, because viability of clutches of ova stripped from a single female on successive days, dropped from 37 % (300 ova) to 6 % (180 ova).

Because there is an inherent tendency for Cyprinella spp. to tank-spawn in the morning, timing of hormonal injections and subsequent surges of hormones (relative to the morning hours) may be critical for ensuring that a high percentage of fish spawn. However, injection schedules in this study were too variable to draw any conclusions regarding injection times and percent ovulation.

Yolk diameters of water hardened ova ranged from 1.01 mm to 1.36 mm, and these diameters correspond to the sizes of mature oocytes competent to respond to hormonal injections. Thus, yolk diameters of Cyprinella spp. ova recorded from clutches of spawned ova in the wild may serve as an indicator of the minimum size

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of oocytes for females selected for hormonal injections. This information should be evaluated in conjunction with that on the minimum size of oocyte that responds to hormonal injections of captive fish.

Time to hatch (based on day on which most embryos hatched) of whitetail shiner embryos ranged from 182 to 230 degree(C)-days. Gale and Buynak (1978) reported that 142 to 190 degree(C)-days are required to hatch embryos of the satinfin shiner, and the number of degree(C)-days to the start of hatching was 108 for the red shiner (Gale 1986), 132 for spotfin shiner (Gale and Gale 1977), and 144 for the Alabama shiner (Ferguson 1990).

The hatching methods used in this study are satisfactory for Cyprinella spp. Most mortalities in the stripping and hatching process were due to clumping of ova at time of stripping. Consequently, larger dishes should be used when spawning rare Cyprinella spp. Nonviable ova and dead embryos should be removed at least once daily to prevent growth of fungus. The first 24 to 36 h are particularly critical because dead organic material is available as substrate for fungus growth at that time. Over-handling of embryos, particularly toward the end of the incubation period, should be avoided because it may cause premature hatching, and premature larvae have a reduced survival rate. A tendency to hatch prematurely when handled was noticed on several occasions while removing dead embryos and while taking photographs of developing embryos. Premature hatching due to over-handling

embryos has also been reported for embryos of the red shiner (Strawn and Hubbs (1956).

Lack of swim bladder inflation of some larvae was noted after hatch of several clutches. This problem is prevalent in the culture of other species, but the underlying cause is unknown. No differences between methodologies for maintaining brood stock, handling fish, injecting fish, stripping ova, or hatching embryos between clutches that had low versus high rates of swim bladder inflation were discernable.

Recommendations for Future Work

This information should be used in conjunction with that already reported in Chapter 1. Wild fish should be acclimated to laboratory conditions for several weeks. They should be fed a daily diet of commercial flake food that is supplemented with a small diameter moist pellet or cultured live food to help ensure good condition of adults and gametes. No structures should be placed in holding tanks in order to facilitate training of fish to accept prepared diets, and cleaning of tanks. Photoperiod should be long (16 h light), and temperature should be between 22 to 24 C. If a warmer temperature is used, it should be reduced prior to handling fish. Frequent observations should be made to ensure that aggressive males are not causing undue harm to other fish.

Tank-spawning of rare Cyprinella spp. is much preferred to induced-spawning because handling-induced stress is alleviated. Consequently, spawning structures

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similar to the type described in this study should be added to the tank, once fish have become acclimated to the laboratory. Fridirici and Beck (1986) have described a technique that is useful for hatching spawned ova of crevice-spawning minnows. Other spawning structures such as stacks of larger slab-rocks or sections of water impregnated logs with attached bark should also be tested as potential spawning substrates, if holding tanks are of a large enough size. These substrates should be sterilized to prevent contamination of tanks with undesirable organisms.

If fish have achieved breeding condition (i.e., females have distended abdomens and prominent ovipositors, and males are highly tuberculated and brightly colored) and they have not tank-spawned after several weeks, or if tank-spawnings have been too infrequent or small to be of use for propagation, hormone induced-spawning should be attempted. Adults should be examined critically for reproductive condition prior to injection. They should be anesthetized ($\approx$ 10 to 25 mg/l MS-222), and oocytes should be sampled from females with a catheter. Males should be examined to see if milt can be stripped, by gently squeezing their abdomen. Salt (0.2 %) and a bactericide can be used in conjunction with an anesthetic to reduce handling stress and help prevent handling-induced bacterial infections. If oocytes of females are at least 1 mm in diameter, then they can be injected; otherwise, they should be returned to the holding tank and reexamined in 5 to 7 d. Males can be injected if they are highly tuberculated and brightly colored.

Either the hCG + CPE or LHRHa + dom treatment at the volumes and concentrations used in this study should be injected, until other studies provide information on more efficacious hormonal treatments. I suggest using the LHRHa + dom treatment because a slightly higher percentage of tame fish spawned following LHRHa + dom injections compared to hCG + CPE injections, and over-ripening of oocytes was less of a problem. I suggest administering the first high dose injection 4 h after the primer injection and injections every 24 h thereafter. The injections should be given intraperitoneally at the base of the pelvic fin with a tuberculin syringe. An antibiotic can be administered at the same time as hormonal treatments to reduce the chance of infection of injected fish. Clemens and Sneed (1962) used 100 units of penicillin per 0.1 mL of hormonal solution for injections of small cyprinids. Females should be checked for ovulation at the time of each injection by gently squeezing the abdomen, but oocytes should not be reexamined until after the third injection (at the time of the fourth injection) to preclude undue stress during the peak time of ovulation and spawning of hormonally-injected Cyprinella spp. At the time of the fourth injection, oocytes should be examined for viability and maturity (i.e., whether germinal vesicle breakdown has occurred). If ova are atretic, injections should be discontinued, and if ova seem competent, then additional injections can be given. The total number of injections should be limited to five to avoid excessive handling of fish, and fish that seem to be stressed should not be given additional

injections. Temperatures of 22 to 24 C seem to be satisfactory for hormonal induction of spawning of <u>Cyprinella</u> spp.

Males should be examined at the time of each injection to determine if milt can be expressed. Availability of milt should not be a problem; however, if males do not become free-flowing after several injections, other hormonal treatments should be tried. Injections of females should be discontinued if males cannot be induced to produce free-flowing milt, unless males can be sacrificed to fertilize ova of stripped females. Aggressive behavior of males toward other fish should be monitored to avoid mortalities. Subdominant males that have been relegated to the surface of the tank should be removed and placed in another tank. Similarly, males overly aggressive to females should be isolated. Spawning structures should be provided at least for initial trials, but the spawning tank must be relatively free of structure to facilitate capture of fish. Spawning structures can be removed prior to capture of fish if they interfere with the netting process.

The diameter of the smallest oocyte to mature as a result of hormonal injections should be determined from oocyte samples collected after hormonal injections. Only females with mean oocyte diameters 0.1 mm larger than the size of those that can respond to hormonal treatments should be selected to receive injections. Yolk diameters of spawned ova should be compared with that of the smallest oocyte that responds to hormonal treatments to further refine the size of ova to use for selection of females.

Wet-stripping should be used for ovulated females, and care should be taken to prevent clumping of stripped ova. Dishes containing fertilized ova should be submerged in rearing tanks and an air stone placed over the dish to provide circulation and aeration. Nonviable ova and dead embryos should be scraped from the spawning dish with a small probe at least twice daily the first two days and daily thereafter. A temperature of 25 to 26 C is satisfactory for incubating embryos of Cyprinella spp. Thirty-eight liter tanks are recommended for hatching and rearing Cyprinella spp. because they are easy to maintain, and can be used in subsequent rearing efforts. Water in incubation tanks should be exchanged several times per day with good quality water.

Additional studies should determine whether larger tanks, low densities of adults, particular sex ratios, or the use of alternative spawning substrates will precipitate more frequent and predictable tank-spawning of hard to spawn species such as the whitetail shiner and the Alabama shiner.

Dosages of hormones used and timing of hormonal injections need to be refined. Suggested strategies include comparison of ability of a hormonal injection sequence initiated in the morning (08:00 to 10:00 h) with one initiated during the evening (16:00 to 18:00 h), to induce ovulation in Cyprinella spp. The use of more frequent (i.e., every 8 or 12 h) injections, and the effectiveness of dosages of hormones, other than those used in this study, should also be investigated. Recommended dosages for preliminary trials are 10 %, 25 %, and 500 % of those

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used in this study. Because larger clutches of oocytes are desirable for propagation, a comparison of clutch sizes of hormonally-induced spawning versus that of natural spawning should be made.

CHAPTER 3

Rearing Larvae of <u>Cyprinella</u> <u>galactura</u>

Introduction

Techniques for rearing larvae of <u>Cyprinella</u> spp. to an advanced fry stage have not been developed. The goal of this study, therefore, was to identify a diet, feeding regime, and rearing system suitable for rearing <u>Cyprinella</u> spp. from the larval stage to an advanced fry stage. Use of a dry diet is preferable to that of live organisms because supplies of prepared food are more dependable, and dry diets are generally more convenient to use. However, the success of rearing cyprinid larvae to an advanced fry stage using only a dry diet varies with species. For example, it is difficult to rear larvae of the common carp, <u>Cyprinus</u> <u>carpio</u>, using only a prepared diet (Dabrowski 1984, Szlaminska and Przybyl 1986), whereas the silver carp, <u>Hypophthalmichthys</u> <u>molitrix</u>, is easily reared on dry diets (Dabrowski 1984). Consequently, use of cultured food organisms and prepared diets to rear <u>Cyprinella</u> spp. was evaluated. The criteria used to evaluate the results of experiments were fish growth and survival. Whitetail shiner larvae were used in all trials, but extrapolation of results of experiments to <u>Cyprinella</u> spp. was made when appropriate.

Materials and Methods

Two tank systems were used to rear larvae in this study. Both used large Living Streams (≈ 500 L) as reservoirs and received a small, steady flow (≈ 1 L per minute) of municipal water. The quality of the water was satisfactory for rearing of larval fishes (Appendix 1). The water entered at an end of the tank and passed through a bed of activated charcoal for dechlorination prior to mixing with water in the main portion of the tank. One system used four 38 L aquaria (four-tank system) and the other used six aquaria (six-tank system), with a resultant total capacity of ≈ 700 L of water in both systems that turned over approximately twice per day (Figure 3.1).

A submersible pump was used to circulate water from the main portion of the reservoir to aquaria, and automatic siphons maintained desired water levels. Brass saddle valves maintained flow rates to aquaria of approximately 2.5 L per minute or four turnovers per hour. This flow rate produced a thin stream of water that sprayed into aquaria. Water siphoned from aquaria was returned to the reservoir. Siphons were sections of 13 mm I.D. clear, flexible vinyl tubing that were screened to prevent escapement of larvae. The screens were 20 cm diameter circles of 363 μm bar mesh Nitex netting that were formed around the inlet of siphons. This provided a relatively large screening area. Holes in the automatic siphons were positioned to maintain tanks at approximately 90 % full (≈ 34 L). Blue-M submersible heaters maintained constant, elevated temperature in all trials.

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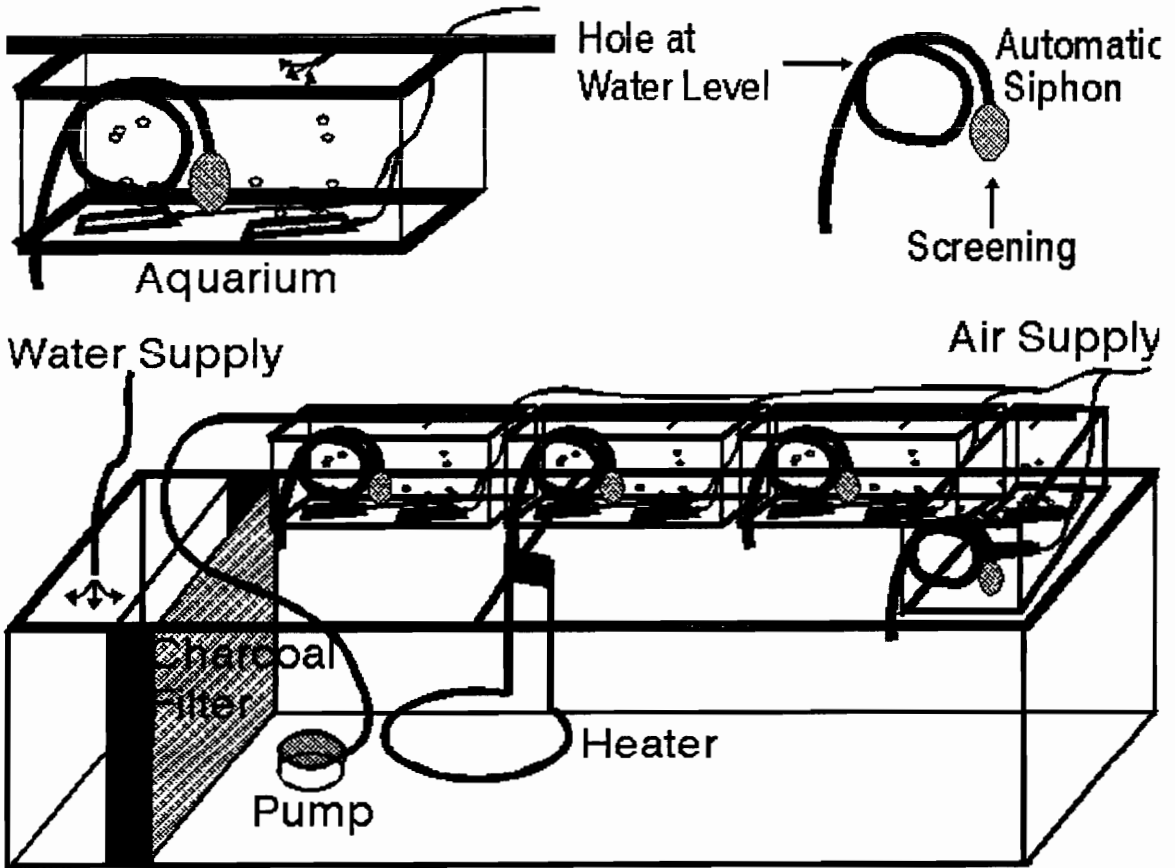
Figure 3.1. Four-aquarium system used in larval rearing trials.

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Larvae used in rearing experiments were obtained from hatches of ova spawned by hormonally injecting whitetail shiners using the procedures described in Chapter 2. Eight trials were initiated, but two trials were prematurely terminated when swim bladders of larvae failed to inflate. The stocking procedure consisted of siphoning larvae from hatching tanks with a section of 6 mm or 13 mm I.D. clear, flexible vinyl tubing. Larvae were siphoned into a 4 L plastic jar with water, and the siphon outlet was submerged to cushion the transfer of larvae from hatching tank to collection jar. Water and captured larvae in the jar were carefully decanted into aquaria for feeding trials. Larvae were counted as they were poured from the jar. This process was repeated until all hatched larvae were harvested and distributed among rearing tanks.

It is possible that less vigorous larvae were captured during initial siphoning efforts, and that larval vigor varied among spawns. Consequently, a conscious effort was made to equally distribute each jar of larvae, and larvae from different spawns among all rearing tanks. Larvae were moved one or two days after the majority had hatched.

Number of larvae used in trials was dependent on clutch size, and hatching success of spawns. In Trial 1, 150 larvae were stocked into each aquarium of the four-tank system. In Trial 2, 65 larvae were stocked into each of four aquaria in the six-tank system. In Trial 3, 55 larvae were stocked into each aquarium of the four-tank system. In Trial 4, aquaria in the six-tank system received 71 to 132 larvae. In

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Trial 5, each aquarium in the six-tank system received 35 larvae, and in Trial 6, each aquarium in the four-tank system was stocked with 30 larvae. Larvae used in Trial 5 and Trial 6 were from the same spawns. All treatments were run in duplicate. To ensure that replicate tanks were not positioned side-by-side, treatments were randomly assigned to aquaria in a consecutive order and that order was repeated for duplicate tanks. For example, in Trial 4, fish in tank 1 were fed a dry diet, fish in tank 2 were fed brine shrimp, and fish in tank 3 were fed a combination of the diets. This order was repeated for tanks 4 to 6.

Feeding trials were conducted at a constant temperature of approximately 25 or 27 C. The photoperiod was 16 h light and 8 h dark. Intensity of lighting was gradually changed over a time period of at least one hour at the beginning and end of each day to preclude startling of larvae. Light intensity was controlled by several timers and a series of incandescent lights (wattages ranged from 5 to 150).

Preliminary results of efforts to develop and maintain cultures of live food organisms (algae and cladocerans) were not satisfactory. The production and subsequent supply of these organisms proved unreliable, and the time required to maintain the cultures was prohibitive. The only live food used in rearing trials were brine shrimp nauplii (Artemia franciscana), because their availability was dependable.

Larvae hatched before the initiation of controlled feeding trials seemed to do well on a diet of finely ground commercial flake food or a microencapsulated diet (AP100, produced by Zeigler Brothers, Inc.) when fed one or two times daily.

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Consequently, larvae were fed twice daily in initial controlled rearing trials. The mean mouth width of several recently hatched whitetail shiners was 470 μm, and consequently, a food particle size discernibly smaller than 470 μm was used in feeding trials. Two microencapsulated feeds were evaluated in rearing trials, Fry Feed Kyowa (250 μm) from Biokyowa, Inc. and AP100 (250 μm). Four of the six completed feeding trials compared AP100 with Kyowa. Dry food was fed at a rate that evoked a strong, sustained feeding response from larvae; this was determined to be approximately 200 mg (6 mg/L) in preliminary trials. Weight of food fed at each feeding was not premeasured; instead, all tanks were fed approximately equal amounts from a container in which the weight of food was known. The mean weight fed per tank per day during each trial was determined by dividing the total weight of food fed during a trial by the number of days in the trial and the number of aquaria that received dry food. Fish were fed by hand once in the morning and once in the evening in five of the six completed feeding trials. In Trial 2, automatic vibrating feeders from Sweeny Enterprises, Inc. were calibrated to feed $\approx$ 33 mg of food hourly for 12 h.

Five grams of brine shrimp cysts were incubated per day at $\approx$ 27 C in a 3 % NaCl solution. Inverted 1 L plastic beverage containers without bottoms were used as incubation vessels. They were supported atop 500 mL beakers in a water bath (a 56 L plastic pan) that was heated by two 100 watt submersible heaters. Water in the bottles was circulated with a strong flow of air from small air stones. Incubation of

new batches of cysts was started at the time of the morning feeding. Nauplii were harvested by removing the air stone, allowing them to settle to the bottom (cysts rise to the top) for several minutes, and then siphoning nauplii from the bottom of the bottle with a section of 6 mm I.D. clear, flexible vinyl tubing. Concentrated nauplii were diluted to 800 mL, swirled to uniformly suspend nauplii in the container, and equal volumes were added to each aquarium that received brine shrimp. Approximately one-third of the volume of brine solution in hatching bottles was siphoned and fed in the morning, and the remainder was used for an evening feeding. Thus, the incubation time was 48 or 60 h, and three hatching bottles were required. The maximum density of larvae fed per tank per feeding was estimated to be 4 nauplii per milliliter (1.25 g cysts * 250,000 cysts per gram * 90 % hatch rate / 2 feedings / 34,200 mL).

Aquaria were siphoned each day before the evening feeding. A plastic straight-edge was slowly scraped along the bottom of tanks to consolidate waste materials prior to siphoning. This procedure greatly reduced the number of larvae, particularly young larvae, that were captured during routine cleanings. The number of dead larvae found in the siphoned waste was recorded in four trials (including one in which swim bladders did not inflate), and live larvae were returned to the appropriate aquarium. Screens were cleaned as necessary.

The number of fish that remained, and length of each fry was determined after 16 or 20 d, except in Trial 1. In Trial 1, all fish that remained after 20 d were

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counted, but lengths were taken only from a subsample of fish. Fish in Trial 1 were reared for an additional 30 d on the same diets used during the first 20 d of the trial. Fry from Trial 4 were reared for an additional 54 d, after assessing their growth and survival in a 16 d feeding trial. They were fed a variety of diets that included commercial flake food, AP100, Kyowa, and occasionally brine shrimp nauplii.

An estimated mean total length of whitetail shiner larvae at the beginning of feeding trials was determined by measuring the lengths of 6 to 15 two or three day old larvae from five spawns with the aid of a binocular scope and ocular micrometer. Total lengths of all fry that remained at the end of each trial were measured to the nearest millimeter, and the mean lengths were calculated for fish in each aquarium. Post-trial mean length values, minus the estimated length of the larvae at the beginning of feeding trials, were divided by the number of days in a trial to compute growth per day. The proportion of larvae that had survived was determined by dividing the number recovered by the number stocked at the beginning of the trial. Survival for each tank was not adjusted for number of days in each trial, but this information was incorporated into interpretation of survival differences among treatment groups. Growth rates and survival values of replicate tanks were averaged, and these values were ranked among all trials. The rankings were used to compare results among all trials, and to select the most appropriate techniques for rearing Cyprinella spp.

Results

Mean temperatures of feeding trials ranged from 24.7 C to 26.7 C. Temperatures did not range beyond one degree of the mean temperature over the course of any trial.

Low densities of brine shrimp were noted in aquaria that were fed only dry diets in Trial 4 and Trial 5. Apparently some nauplii were transported to the reservoir in overflow water from aquaria that received brine shrimp, and were then pumped into all aquaria. Thus, fish larvae fed only dry food inadvertently received a supplement of brine shrimp. Densities of nauplii in aquaria that were fed only dry food were not measured, but they were considerably lower than those in tanks that received direct feedings.

Brine shrimp remained in the water column (and subsequently were vulnerable to predation by larvae) for several hours after each feeding. Dry food initially floated on the surface of the water and eventually sank to the bottom. It was available to larvae as it sank. The concentration of food in the water column was relatively dense immediately after feeding and gradually declined thereafter. It took approximately 15 to 20 min for all dry food to disappear from the surface after each feeding. Larvae fed only infrequently on food on the aquarium bottom.

Amount of dry food fed per feeding per tank ranged from 200 to 310 mg for all trials, except Trial 2. In Trial 2, the mean weight of dry food per feeding ranged from 30 to 50 mg, and the variance was extremely high. Sweeny automatic vibrating

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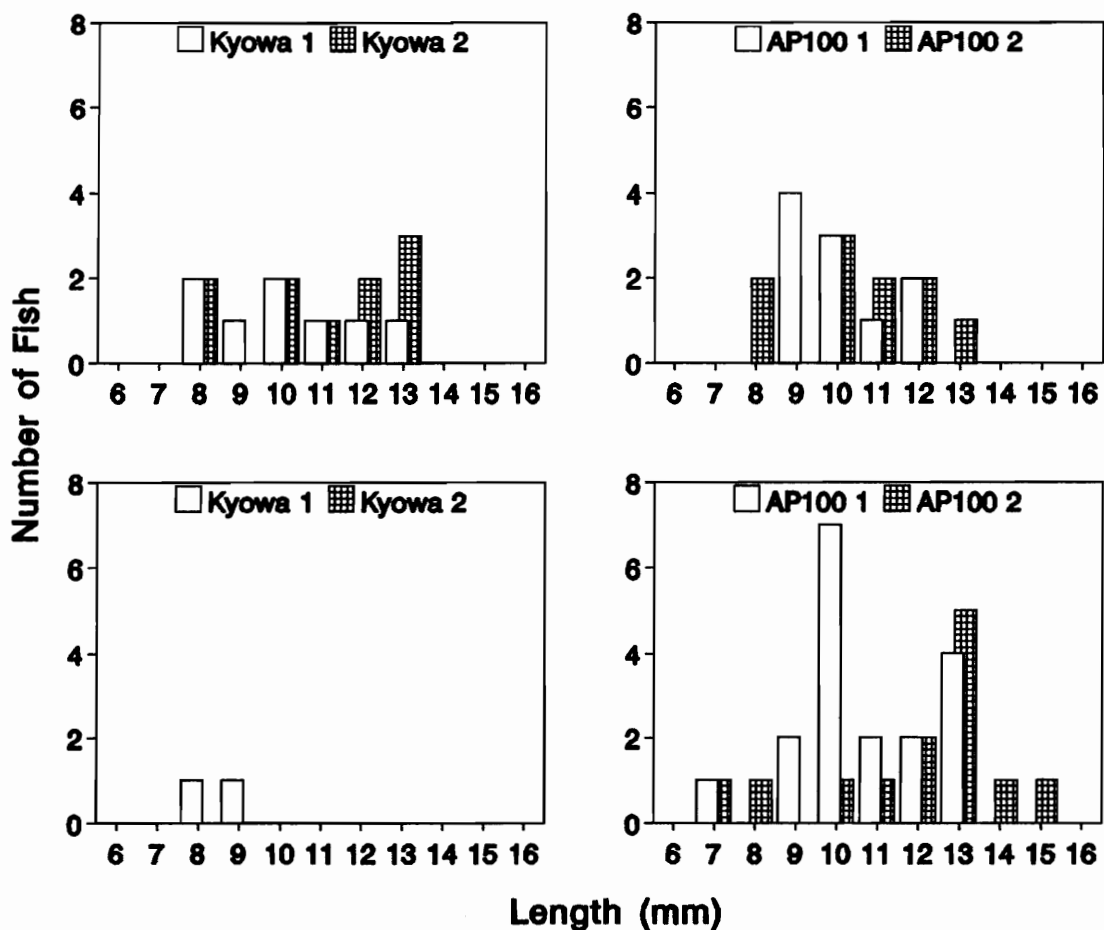
Figure 3.2. Total lengths (mm) of <u>Cyprinella</u> <u>galactura</u> fry fed Kyowa or AP100 (top graphs - Trial 1, bottom graphs Trial - 2) in 20 d feeding trials.

feeders were used in this trial. Even with constant adjustments, these feeders were unable to consistently deliver the small quantities of food fed to larvae.

Growth of fish in duplicate tanks was similar for all treatments in all trials (Figures 3.2, 3.3, and 3.4). Except for Trial 1, the numbers of fish used in the length frequency distributions of Figures 3.2 to 3.4 represent the number of fish remaining at the end of each trial. In Trial 1, 27 of the fish fed Kyowa remained, and 86 of the

120
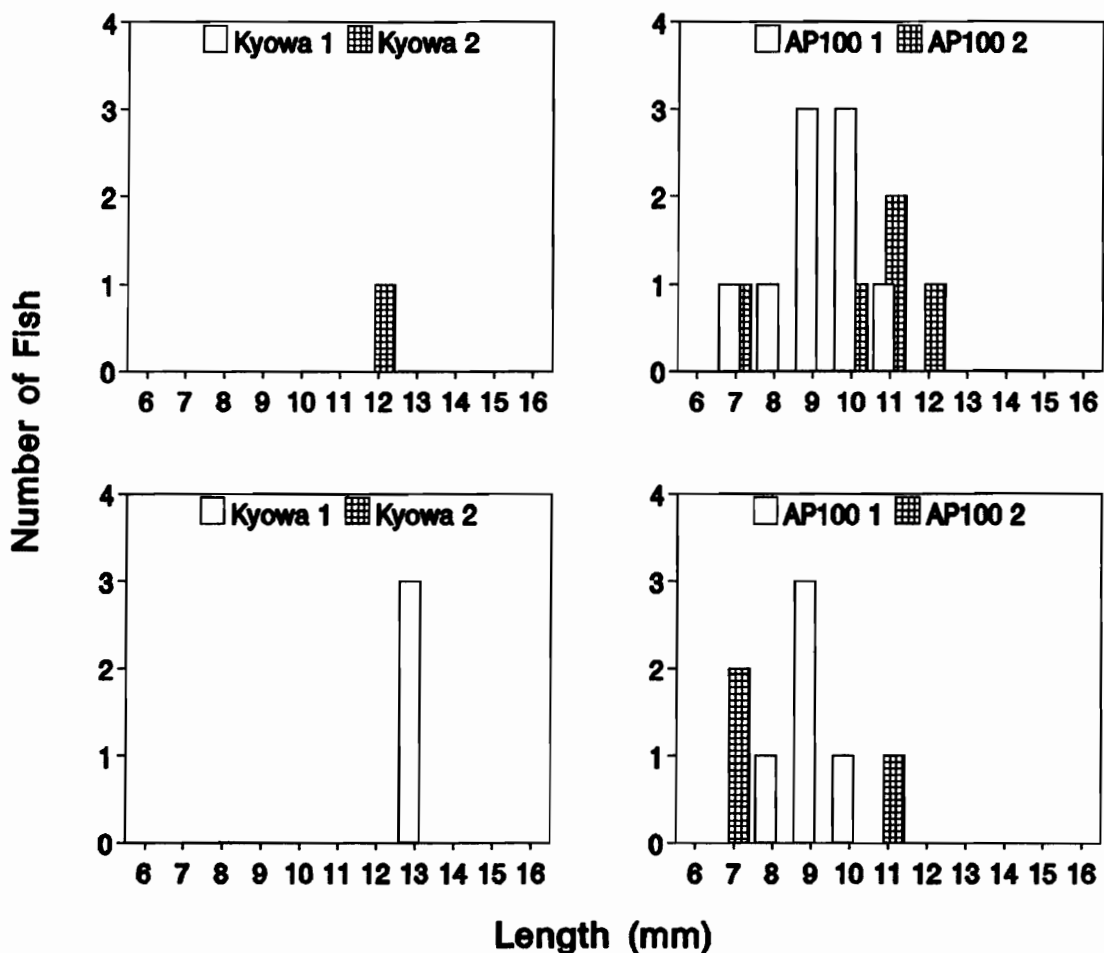
Figure 3.3. Total lengths (mm) of Cyprinella galactura fry fed Kyowa or AP100 (top graphs - Trial 3, bottom graphs Trial - 6) in 16 d feeding trials.

fish fed AP100 remained.

Mortality of fish in duplicate tanks was also similar for all treatments and trials. Mortality of a batch of larvae in which most fish lacked swim bladders was followed for 15 d (Figure 3.5). Most of these fish died between day 5 and day 10, and the few that survived after that time had swim bladders. In Trial 6, the mortality pattern resembled that of fish that lacked swim bladders. The main period of

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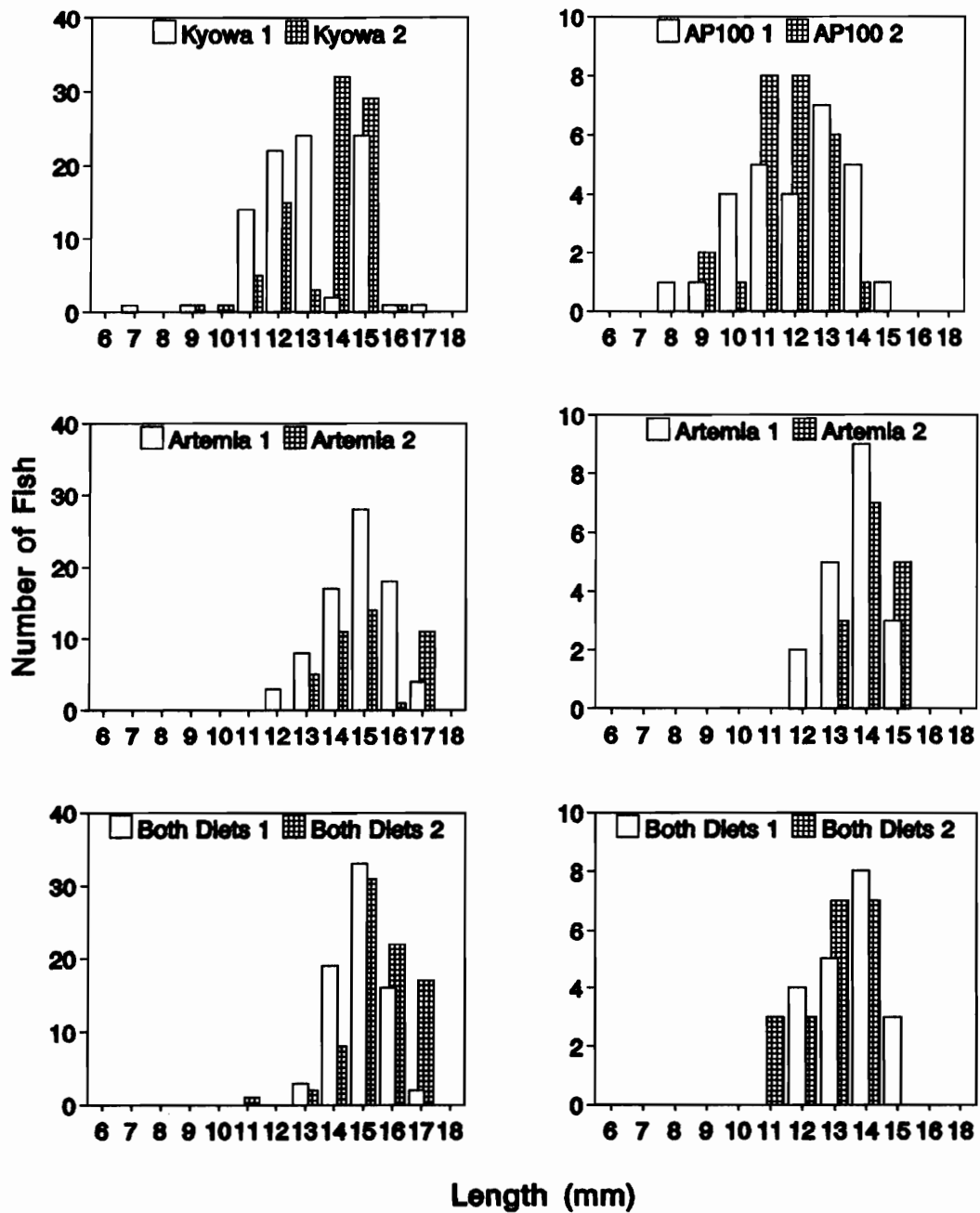
Figure 3.4. Total lengths (mm) of <u>Cyprinella</u> <u>galactura</u> fry fed Kyowa, <u>Artemia</u>, or Kyowa plus <u>Artemia</u> (left graphs - Trial 4), or AP100, <u>Artemia</u>, or AP100 plus <u>Artemia</u> (right graphs - Trial 5) in 16 d feeding trials.
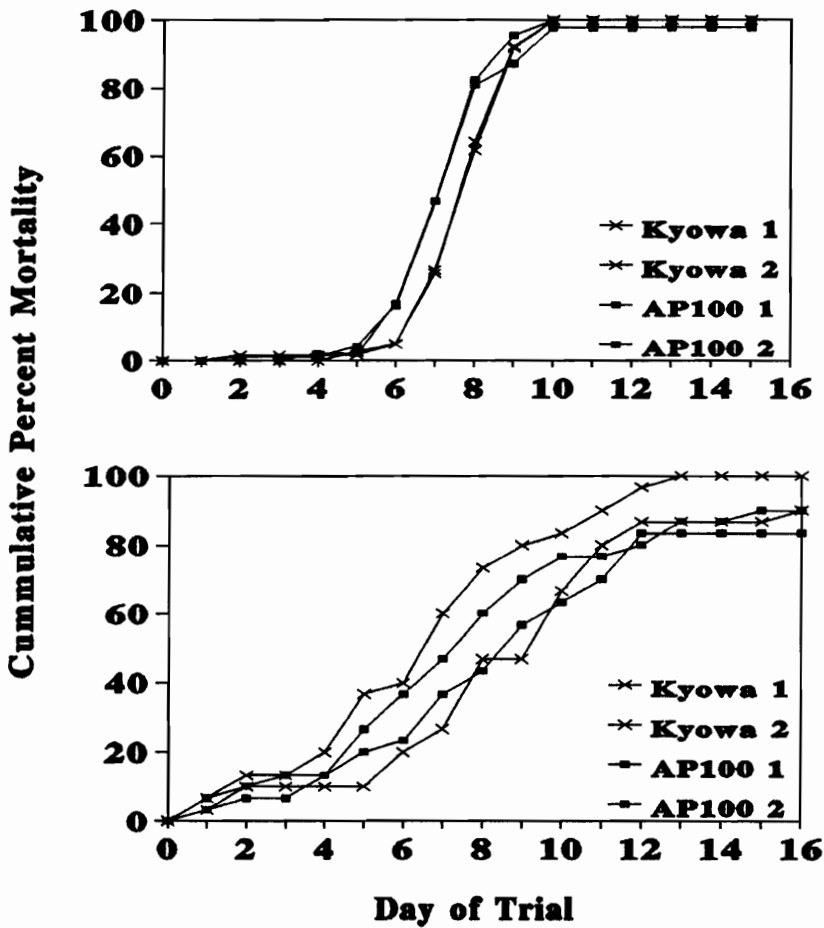
Figure 3.5. Mortality of <u>Cyprinella galactura</u> fry that lacked swim bladders (top graph) over a 15 d time period, and of fry that were fed Kyowa or AP100 (bottom graph - Trial 6) in a 16 d feeding trial.

mortality was slightly longer, and most fish died between day 5 and day 12 (Figure 3.5). This pattern of mortality was the same for all feeding trials in which only dry diets were fed (Trials 1, 2, 3, and 6). Mortality of fry in Trial 4 was similar for all treatments; episodes of mortality were noted from day 0 to day 2, and from day 9 to day 11 (Figure 3.6). Mortality of fry in Trial 5, like that in Trial 4, consisted
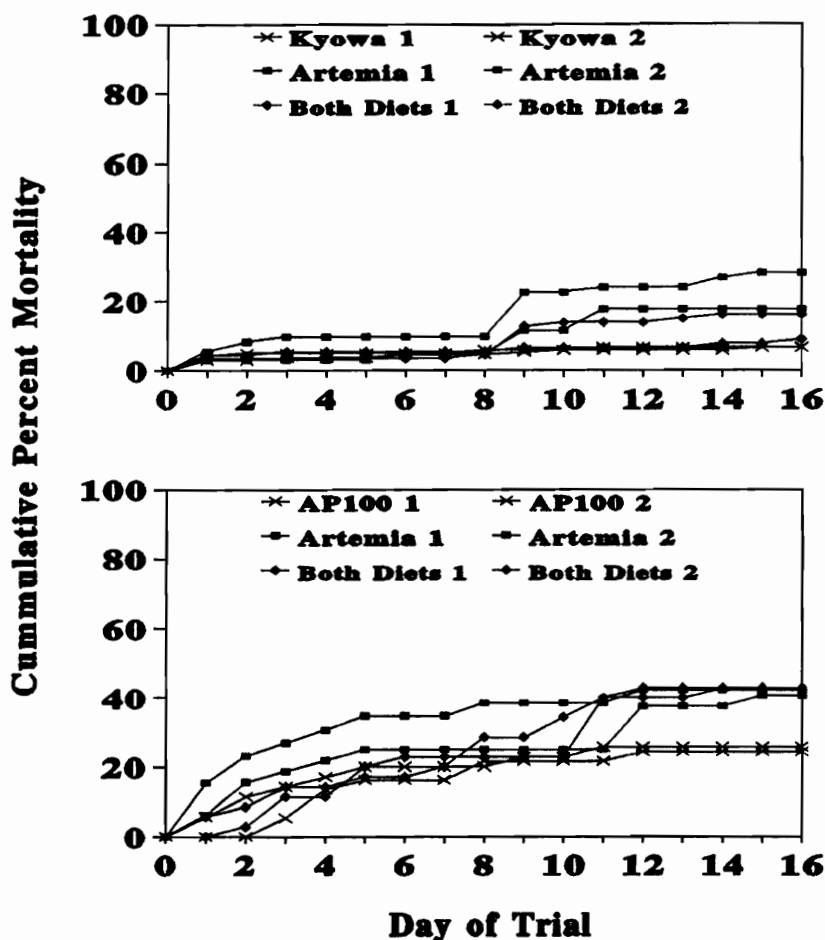
Figure 3.6. Mortality of Cyprinella galactura fry fed a diet of Kyowa, Artemia, or both diets (top graph - Trial 4), and of fry fed AP100, Artemia, or both diets (bottom graph - Trial 5) in 16 d feeding trials.

of two distinct episodes. The first period of mortality lasted from day 0 to day 4, and the second from day 8 to day 12.

Growth and survival of whitetail shiner larvae were lowest in trials with only dry diets (Table 3.1). Survival ranged from 0 to 13 %, and mean lengths ranged from 8.5 to 13.0 mm for larvae fed only Kyowa. Survival of fish fed only AP100 ranged

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Table 3.1. Comparison of growth and survival of <u>Cyprinella</u> <u>galactura</u> fry among all feeding trials.

Trial ID length - d (temperature - C)	Diet	Amount fed per day (feedings per day)[1]	Mean TL ± SD[2] per tank	Percent survival (number of fish)[3]	Rank G+S[4]
Trial 4 16 (25.5)	Kyowa + <u>Artemia</u>	490 mg of dry	14.9 ± 0.9 15.5 ± 1.1	93 (87) 84 (89)	1+1
	<u>Artemia</u>	1.25 mg cysts (2)	14.8 ± 1.2 15.2 ± 1.3	93 (96) 78 (71)	2+3
	Kyowa[5]		13.1 ± 1.6 13.7 ± 1.4	93 (116) 82 (132)	4+2
Trial 5 16 (24.8)	<u>Artemia</u>	490 mg of dry	13.7 ± 0.9 14.1 ± 0.2	54 (35) 43 (35)	3+6
	AP100 + <u>Artemia</u>	1.25 mg cysts (2)	13.5 ± 1.0 12.9 ± 1.0	57 (35) 57 (35)	5+5
	AP100[5]		12.0 ± 1.7 11.7 ± 1.2	80 (35) 74 (35)	6+4
Trial 3 16 (25.5)	AP100	400 mg (2)	9.2 ± 1.1 10.2 ± 1.7	16 (55) 13 (55)	7+9
	Kyowa		-- 12.0 ± 0	0 (55) 2 (55)	NR[6]+13
Trial 2 20 (26.7)	AP100	350 mg to 600 mg	10.7 ± 1.6 11.9 ± 2.2	28 (65) 20 (65)	8+8
	Kyowa	(12)	8.5 ± 0.5 --	2 (65) 0 (65)	NR+13
Trial 1 20 (25.3)	AP100	500 mg (2)	9.8 ± 1.3 10.1 ± 1.4	35 (150) 23 (150)	10+7
	Kyowa		9.9 ± 1.8 10.6 ± 1.8	5 (150) 13 (150)	9+11
Trial 6 16 (24.7)	AP100	630 mg (2)	9.0 ± 2.6 7.0 ± 1.9	17 (30) 10 (30)	10+10
	Kyowa		13.0 ± 0 --	10 (30) 0 (30)	NR+12

[1]Weight of cysts is weight of <u>Artemia</u> cysts prior to incubation
[2]Total length (mm) of fish ± one standard deviation
[3]Number of fish at beginning of trial
[4]Rank of mean growth per day (G) and survival (S) for each treatment over all trials
[5]Received some <u>Artemia</u> from reservoir
[6]Not ranked due to lack of replication (i.e., 0 % survival in replicate tank)

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from 10 to 35 % and mean lengths ranged from 7.0 to 11.9 mm. The greatest growth and survival rates were obtained with a diet of Kyowa plus brine shrimp (0.95 mm/d and 88 %). The next two highest growth rates were obtained with a diet of brine shrimp only in Trial 4 and Trial 5 (0.94 mm/d and 0.89 mm/d).

Mean total lengths of Trial 1 fish after 50 d was 18.3 ± 4.5 mm for fish fed AP100, and 26.7 ± 4.6 mm for fish fed Kyowa. Survival during the 30 d post-trial period was 67 % for fish fed AP100 and 48 % for fish fed Kyowa. These values are underestimates because some measured on day 20 died from handling. Mean total length of a subsample of Trial 4 fish (N=30) after 70 d was 33.9 ± 2.9 mm. Mortality was not monitored, but was very low after mortality induced by handling had ceased.

Discussion

Twice daily feedings of AP100 or Kyowa are not satisfactory for rearing *Cyprinella* spp. Twelve feedings per day of the dry diets per day may also be ineffective based on the results of Trial 2, but the validity of this test is suspect due to the inability of the feeders to accurately dispense desired food quantities during the trial. Survival of whitetail shiners fed only AP100 was higher than those fed only Kyowa in all trials that compared the diets, but growth was generally better for fish fed Kyowa.

The highest survival of fish fed only dry diets (mean for treatment tanks = 29 %) was considerably lower than for treatments in which all or part of the diet included brine shrimp. The pattern of mortality approximated that of fish without inflated swim bladders implying that death of larvae that were offered only dry diets was due to starvation of nonfeeders. Growth rates of fish fed only dry diets was also consistently lower than those of fish that received some brine shrimp nauplii in their diet. The poor performance of fish in Trial 6 compared to those in Trial 5 provides very strong evidence that growth and survival of *Cyprinella* spp. larvae is enhanced by incorporating brine shrimp nauplii in the diet, because fish larvae used in both trials were from the same spawns.

Use of dry diets typically entails more than two feedings per day. For example, Rottmann and Shireman (1991) fed grass carp, *Ctenopharyngodon idella*, bighead carp, *Hypophthalmichthys nobilis*, and silver carp larvae eight times per day;

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Dabrowski (1984) fed common carp, grass carp, silver carp, and bighead carp larvae 15 times per day; and Charlon and Bergot (1984) fed common carp larvae 288 times per day. Larvae of species difficult to rear on a dry diet such as the striped bass, Morone saxatilis, have been fed every three hours (8 times per day, Webster and Lovell 1990), and larvae of the walleye, Stizostedion vitreum, have been fed every 3.5 min (411 times per day, Barrows et al. 1988) or every 5 min (288 times per day, Loadman et al. 1989). The total weight of food dispensed per liter in the feeding experiments that employed $\geq$ 288 feedings per day ranged from 353 mg/L to 947 mg/L. The weights of food fed in this study were approximately a tenth of those values. An increase in number of feedings and quantity of food fed may increase the survival and growth of Cyprinella spp. larvae fed only a dry diet, but this would necessitate the development of a more elaborate rearing system (Charlon and Bergot 1984) or more frequent cleaning of tanks to ensure maintenance of adequate water quality. A more elaborate system lends itself to a higher chance for failure, and more frequent cleaning can result in higher coincidental loss and mortality of larvae. A higher potential for poor water quality, commensurate with feeding rate, also exists when greater amount of food are used. All are undesirable from the perspective of rearing larvae of rare fishes. Consequently, use of dry diets only to rear larvae of Cyprinella spp. is discouraged, because inclusion of brine shrimp nauplii into the feeding regime for larval whitetail shiners resulted in good growth and survival.

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The best survival and growth was obtained in Trial 4 by fish that were fed Kyowa and brine shrimp. Fish in all treatment groups in Trial 4 exhibited good growth and survival. Survival and growth of fish in Trial 5 was also good. The first episode of mortality in both trials is attributed to poststocking mortality, and the second is attributed to mortality of nonfeeders.

Direct comparisons of growth and survival among trials is complicated by apparent variations in vigor among batches of larvae. For example, survival and growth rates of larvae fed only brine shrimp in Trial 5 were less than those of fish fed only brine shrimp in Trial 4, implying that the larvae used in Trial 5 were less vigorous than those used in Trial 4. Consequently, the relative effectiveness of AP100 plus brine shrimp nauplii versus Kyowa plus brine shrimp nauplii as food for larvae of Cyprinella spp. cannot be inferred from the feeding trials conducted in this study.

In Trial 5, the survival of larvae fed AP100 plus an inadvertent supplement of brine shrimp was notably greater than that of fish that received either brine shrimp only or direct feedings of brine shrimp plus dry food. The best growth, however, was exhibited by fish that received only brine shrimp. Larvae fed a diet of brine shrimp plus dry food had intermediate values of growth and survival in Trial 5. The reason for the higher survival of larvae that were fed AP100 with incidental supplements of brine shrimp is not known. The only obvious difference among treatment tanks was the daily accumulation of additional waste (brine shrimp cysts) in tanks that were directly fed brine shrimp. The growth of fish in Trial 4 and Trial 5 was lowest for

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those fish that received direct feedings of only dry diets; nonetheless the high survival of larvae in both trials suggests that even low densities of brine shrimp fed twice daily can dramatically enhance survival of larvae fed dry diets.

A diet of brine shrimp alone is satisfactory for rearing larval Cyprinella spp. to an advanced fry stage. However, addition of a dry food to the diet (either AP100 or Kyowa) helps to ensure a more balanced diet. Use of a dry food in the early stages of rearing also serves as a precaution against hatch failure of a batch(es) of brine shrimp nauplii, and very likely aids in the transition of advanced fry from a diet that contains live food to one that is wholly or mostly composed of prepared diets. This transition occurs at approximately 14 mg (13 d old) or 18 mg (13 mm long) for common carp larvae according to Bryant and Matty (1981) and Dabrowski (1984).

Controlled experiments to investigate the size at which Cyprinella spp. larvae can successfully be switched from a diet that includes brine shrimp to a diet of mostly dry food were not run, but the relatively good survival of fish in Trial 1 fed only a dry diet after 20 d suggest that it is $\le$ 20 d. Growth rate of these fish was 0.37 mm/d for fish fed AP100 and 0.53 mm/d for fish fed Kyowa. Fish from Trial 4 were fed AP100, Kyowa, commercial flake food, and occasionally brine shrimp for 54 d after the larval rearing trial and their growth rate was 0.48 mm/d. Many fry from Trial 4 died within a day or two following transfer to a holding tank as a result of handling to obtain length measurements. Subsequent mortality of these fish was minimal. These fish were relatively crowded, but seemed to be in excellent condition. Thus, a

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combination of dry diets or a diet of only Kyowa seems satisfactory for rearing Cyprinella spp. fry > 16 d old and juveniles.

The tank systems used to rear larvae in this study proved extremely reliable. The influx of fresh water maintained extremely good water quality throughout all feeding trials, even when the highest concentrations of dry food were fed (maximum total ammonia < 0.2 mg/L, nitrite undetectable, and O_2 > 7 mg/L). The large reservoir is considered essential because it permits dilution of waste products and allows mixing of fresh water with aged water. A turnover of approximately two water exchanges per day permitted maintenance of an equilibrium of important water chemistry characteristics such as temperature and pH ($\approx$ 8.09 in this study); exchange of a large volume of water in a short time period may not. Placement of aquaria over the reservoir is beneficial in that overflow of tanks as a result of clogged screens or air locks in siphons drains into the reservoir, and this permits normal functioning of the rest of the system. Air locks can be avoided if air stones are kept away from the siphon inlet. Clogging of screens can also be easily prevented by replacing screens that have begun to biofoul ($\approx$ 1 week) with new screens. Rearing tanks should be cleaned at least once a day before the last feeding. Removal of waste at this time helps ensure good water quality and buffers against possible failure of the air supply or water supply during the night.

The activated charcoal filter helped to ensure that slugs of chlorine or other undesirable chemicals from municipal water supplies do not enter rearing tanks. It

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also serves to directly remove toxic waste products produced by larvae and decomposition of food. The filter may have a secondary water cleansing function as a substrate for nitrifying bacteria.

The large reservoir and rapid turnover of water in aquaria serve to buffer against rapid changes in temperature in the event of a heater malfunction. The spraying of pumped water into rearing tanks facilitated the feeding of dry food by gradually, but steadily, causing the food to sink. Placement of air stones directly beneath the inlet seemed to lengthen the time period over which food was suspended and the subsequent time of availability of dry food to larvae.

The simple brine shrimp hatching system also worked extremely well. Poor hatches sometimes occurred if cysts accumulated below air stones that were off the bottom. Thus, air stones used to circulate water in the hatching bottles must be small enough to extend into the neck of the bottle, and they should be placed in the very bottom of the neck to ensure agitation of all cysts and a high hatch rate.

Recommendations for Future Work

A tank system similar to that described in this study should be used to rear larval Cyprinella spp. because the design is simple, effective, and reliable. Duplicate systems should nonetheless be used for rare Cyprinella spp. to guard against loss of all fish in the event of a system failure. Parallel parts to systems, such as pumps and heaters, are not necessary because the system can function satisfactorily for $\geq$ 12 h

following a malfunction of one or more components. However, back-ups for all parts (tanks, heaters, pumps, air compressors) should be available. No other fish should be held in the system, and it should be isolated from tanks of other fish to prevent transfer of disease organisms. As an added precaution against disease, the system should be sterilized prior to rearing larvae, and equipment used in the rearing process should not be interchanged among systems.

Spawns of rare Cyprinella spp. should be hatched in rearing tanks to preclude mortality induced by transfer of larvae between tanks. The primary conclusion drawn from this study is that Cyprinella spp. larvae should be reared on a diet of brine shrimp nauplii plus dry food. Kyowa and AP100 are suitable dry diets.

Growth and survival of whitetail shiners in Trial 4 and Trial 5 of this study was good, but feeding rates higher than those used in this study may be more effective for rearing Cyprinella spp. larvae. Additional rearing experiments should be carried out to determine if a greater number of feedings can enhance growth or survival of Cyprinella spp. larvae. It is possible that feeding more than twice per day will enhance growth and perhaps survival of larval Cyprinella spp. Consequently, I recommend investigating the effectiveness of higher feeding rates on rearing larvae and fry of Cyprinella spp. Suggested feeding regimes for further studies are 20 nauplii per milliliter per day plus 20 mg/L/d of dry food fed uniformly over four daily feedings, 20 nauplii per milliliter per day plus 40 mg/L/d of dry food fed uniformly over eight feedings per day, and 10 nauplii per milliliter per day fed twice daily plus

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20 mg/L/d fed of dry food fed uniformly over eight feeding per day. These should be compared to a regime similar to the successful one used in this study; 10 nauplii per milliliter per day plus 10 mg/L/d of dry food fed uniformly over two feedings per day is suggested. The effectiveness of AP100 and Kyowa as dry diets to propagate larvae and fry of Cyprinella spp. should be directly compared. In this study, AP100 generally produced better survival than Kyowa, but Kyowa generally produced better growth than AP100.

Gains in growth or survival that result from increases in feeding rate must be balanced against requisite increases in number of tank cleanings and overall effects on water quality. The likelihood for massive die-offs of reared larvae increases proportionally to the potential for rapid water quality deterioration. Effects of diet and rearing conditions on survival of rare Cyprinella spp. should be weighed more heavily than their effects on growth.

Higher or more frequent feeding rates should not be used on rare fishes until studies, similar to those listed above, have been conducted on surrogate species. Until further studies have evaluated the efficacy of higher feeding rates, I recommend use of Kyowa plus brine shrimp because survival of larvae in Trial 4 was exceptional. Brine shrimp should be fed at a minimum rate of 8 nauplii per milliliter per day and Kyowa at 12 mg/L/d. The diets should be fed simultaneously once in the morning and once during the evening. Rearing tanks should be cleaned prior to the evening feeding. If rare Cyprinella spp. are reared, duplicate water baths to incubate brine

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shrimp cysts should be used to guard against hatch failures. Temperature should be maintained at approximately 25 C in rearing trials. Use of less than 12 mg/L of dry food per feeding is not recommended because lesser amounts do not always evoke a strong, sustained feeding response by larvae. The concentration of dry food must be relatively high for several minutes because larvae have a very limited sphere of activity.

Growth and survival of fry older than 20 d was not critically evaluated. Recommended studies include controlled rearing trials to evaluate growth and survival of older fish fed diets of commercial flake food supplemented with AP100, Kyowa, or salmon starter. Commercial flake food is recommended as the base diet because it has proven satisfactory for development and maintenance of spawning condition of adult Cyprinella spp. These fish should be fed two or three times per day ad libitum. Feeding durations of five to ten minutes are probably adequate. If survival is not satisfactory a live food (e.g. brine shrimp) should be included in the diet, because it may encourage a stronger feeding response and reduce the number of nonfeeders. These trials should also be conducted at approximately 25 C.

CHAPTER 4

Control of gonadal maturation of <u>Noturus</u> <u>insignis</u>

Introduction

Control of gonadal maturation is requisite to controlled spawning of captive fishes. Commercial producers of ictalurids rely on natural maturation of gonads and subsequent spawning of pond-reared fish during the spring and early summer (Tucker and Robinson 1990). Several ictalurids have spawned out-of-season in aquaria (Smith and Harron 1904, Breder 1935, Fontaine 1944), but out-of-season maturation has been critically evaluated only for the channel catfish, <u>Ictalurus</u> <u>punctatus</u>, (Brauhn 1971, Brauhn and McCraren 1975). There is only one report on out-of-season maturation and spawning of madtoms, <u>Noturus</u> spp. (Bowen 1980). The goal of this research was to develop a method to induce gonadal maturation of captive madtoms by using the margined madtom, <u>Noturus</u> <u>insignis</u>, as a model species. Four investigations (objectives) were conducted to achieve this goal. They are listed and described below.

Temperature-Photoperiod Experiments

Temperature and photoperiod are the two primary factors controlling maturation of fish gonads in temperate regions (de Vlaming 1972, Crim 1982). Out-

of-season maturation of captive fish, and subsequent out-of-season spawning, can be achieved through manipulation of these environmental cues (Harrington 1950, Horvath 1986). The purpose of this study was to assess the effects of several combinations of modified temperature and photoperiod cycles on maturation of the gonads of the margined madtoms.

Description of Gonads and Gametes

Clugston and Cooper (1960) described the gonads of the margined madtom, but the gonads and gametes of female and male Noturus spp. have not been critically examined. Consequently, gonadal and gametic characteristics relevant to assessment of state of gonadal maturation, and facilitation of spawning of madtoms were examined. The objective of this study was to compare the morphology and biology of the gonads and gametes of the margined madtom with those of other ictalurids.

Plasma Steroid Levels

Steroids involved in the reproductive cycle of madtoms have not been identified. At the onset of vitellogenesis (this occurs several months prior to the spawning season in temperate fishes) an increase in plasma estrogen levels, mainly 17β-estradiol, occurs in the female of many teleosts (Shreck and Hopwood 1974, Wingfield and Grimm 1977, Crim and Idler 1978, Bromage et al. 1982). The hormone, 17β-estradiol, is produced by maturing follicles (Kagawa et al. 1982), and

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it induces the synthesis and secretion of vitellogenin (Wallace and Selman 1981). Low levels of plasma estrogens are associated with the end of the sexual cycle, and can be very low prior to final maturation, ovulation, and spawning of oocytes (Wingfield and Grimm 1977, Fostier et al. 1978, Sower and Shreck 1982).

In the male of many teleosts, rising testosterone levels are correlated with testicular recrudescence. Peak plasma concentrations of testosterone occur just prior to the breeding season (Campbell et al. 1976, Simpson and Wright 1977, Wingfield and Grimm 1977). The hormone, 11-ketotestosterone is quantitatively predominant to testosterone in several species of fish (Schmidt and Idler 1962, Idler et al. 1971, Campbell et al. 1976, Campbell et al. 1980), but testosterone levels peak just prior to 11-ketotestosterone levels because it is one of the intermediate products in the synthesis of 11-ketotestosterone (Scott et al. 1980, Matty 1985). Therefore, blood concentrations of either steroid can be used to monitor seasonal hormonal changes that correlate with reproductive condition of the male for many fish species.

Objectives of this study were to determine whether 17β-estradiol could be detected in the plasma of female margined madtoms, and whether testosterone could be detected in the plasma of male margined madtoms. If detection was possible, further objectives were to assess the relationship between concentrations of each of the steroids and the stages of gonadal maturation, and to compare levels of the hormones produced by captive fish with those produced by wild fish.

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Multiple Injections of CPE

Strip-spawning of ictalurids requires sacrifice of the male because milt cannot be obtained by squeezing the abdomen of ripe males. Enlargement of the testes, an increase in number of spermatozoa per testis, or most importantly, assurance of good quality sperm from males could reduce the number of males that must be sacrificed to fertilize ova from strip-spawned females. Injections of hormones have been used to promote spermiation and increase milt volume of several species of fish (Belova 1981, Weil et al. 1986). The objective of this study was to determine the effects of multiple injections of carp pituitary extract (CPE) on the testes and spermatozoa of margined madtom males.

Materials and Methods

Fish used in all experiments were collected by back-pack electrofishing in either Stroubles Creek (Montgomery County), Toms Creek (Montgomery County), or Wolf Creek (Bland County). All are tributaries to the New River, Virginia. All wild fish examined in the photoperiod-temperature study were collected from Stroubles Creek. The fish were prophylactically treated for disease for 3 to 4 h with salt (0.2 %), formalin (25 mg/L), and Furacin (10 mg/L) immediately after capture and periodically thereafter.

Captive fish were fed a diet of moist pellet once or twice daily. The moist pellet was prepared by grinding whole, fresh or frozen fish with commercial trout or catfish feed in a commercial meat grinder equipped with a 3 mm extruder plate.

In some experiments the spawning condition of females was judged by externally discernible characteristics, such as a greatly distended abdomen or large oocytes. The outline of large oocytes of <u>Noturus</u> spp. can be detected through the abdominal wall when the abdominal wall tissue located just anterior to the vent is slowly moved back-and-forth.

Tanks used in experiments included 110 L aquaria, 500 L (2.1 X 0.5 X 0.4 m) rectangular fiberglass tanks (Living Streams), and screened-off sections of a 7000 L (17.0 m circumference X 1.2 m width X 0.4 m deep) oval fiberglass raceway. Twelve aquaria were used in a recirculating system. Each aquarium in the twelve-tank system was equipped with an under-gravel filter that served to remove waste products

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and provide aeration. Water from all aquaria drained into a common reservoir (a Living Stream) from which it was pumped back into aquaria. This system received small, constant inputs of well water. The turnover rate was not determined for this system, but it was estimated to be one or more reservoir volumes per day. The other tanks used (Living Streams and the oval raceway) received inputs of municipal water that were filtered through a bed of activated charcoal for dechlorination. The volume of water in the Living Streams turned over approximately twice daily, and that of the oval raceway approximately every two days.

Temperature-Photoperiod Experiments

Photoperiod was controlled by several timers and incandescent lights, or by a computer-controlled dimmer and incandescent lights. In both systems, the lighting intensity was gradually changed over a period of at least one-half hour at the beginning and end of each daylight period. The gradual change was included to preclude startling and subsequent stressing of fish used in experiments. Natural photoperiod for each day was calculated by adding one hour of light (i.e., a 0.5 h dawn and 0.5 h dusk period) to lengths of days (sunrise to sunset) listed in The Farmer's Almanac. The times were corrected for latitude.

Water temperature was controlled by Frigid Unit water chillers (Living Streams), inputs of heated or unheated well water (aquaria system), adjustment of room temperature within an environmental chamber (Living Streams), or with a large

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refrigeration unit attached to the oval raceway. The environmental chamber was a 2.4 m X 2.4 m insulated room in which air temperature was thermostatically regulated via use of air-conditioning or heating units. Heating of water was required only in the system that used well water, because the warmest temperature did not exceed ambient room temperature.

The ability of margined madtoms to develop mature gonads under controlled conditions was evaluated in a series of experiments that used five combinations of temperature and photoperiod. Temperature and photoperiod were manipulated in four ways; 1) natural cycles were simulated, 2) natural cycles were compressed from time of capture to a time when expected natural spawning conditions were reached, 3) natural cycles were compressed, but began with mid-winter conditions and then continued to a time when expected natural spawning conditions were reached, and 4) temperature was held constant at a level characteristic of the natural spawning season. Natural spawning conditions were considered to be 15 to 16 h light and 22 to 23 C. The combinations of temperature and photoperiod were as follows:

1) WF - wild fish under a natural photoperiod and temperature regime (Figure 4.1). The first collection of fish was in early November.

2) NT-NP - captive fish held under simulated natural conditions (Figure 4.2). Fish were collected and confined in late September.

3) UET-CWP - captive fish held under uniform, elevated temperature and a compressed photoperiod cycle that started with a mid-winter photoperiod

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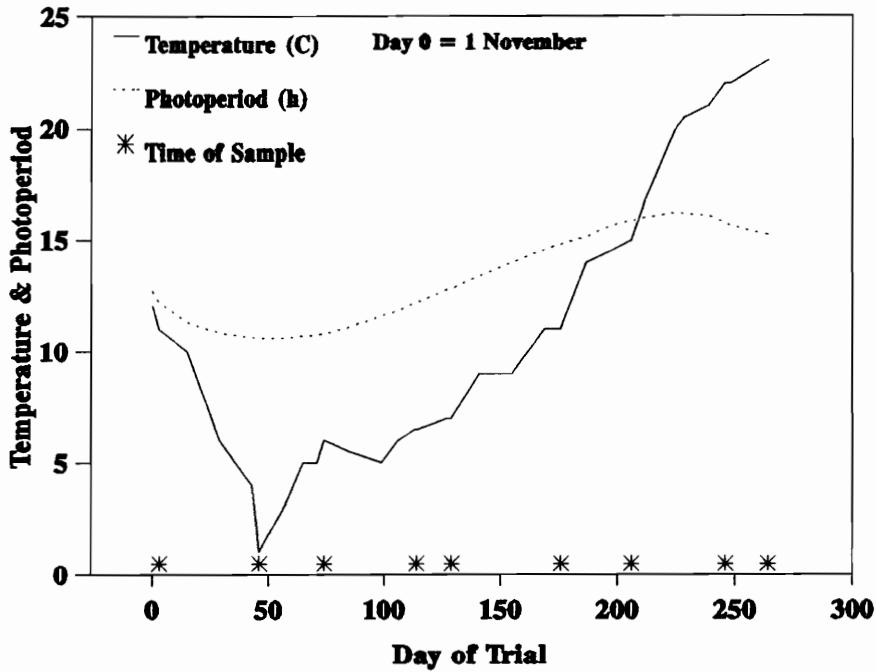
Figure 4.1. Approximate temperature and photoperiod regimes for fish collected from the wild.
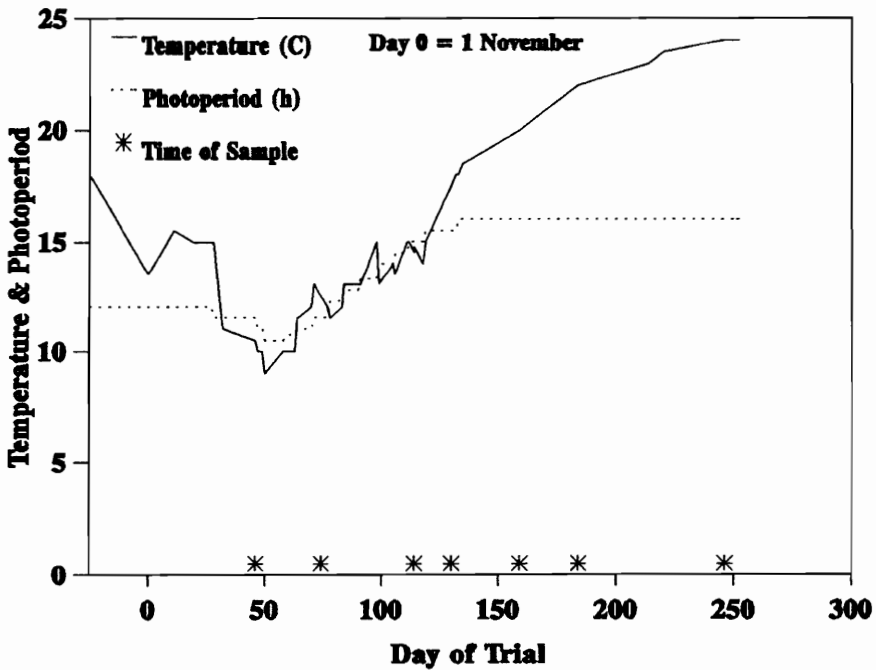
Figure 4.2. Approximate temperature and photoperiod regimes for captive fish held under simulated natural temperature and photoperiod (Experiment NT-NP).

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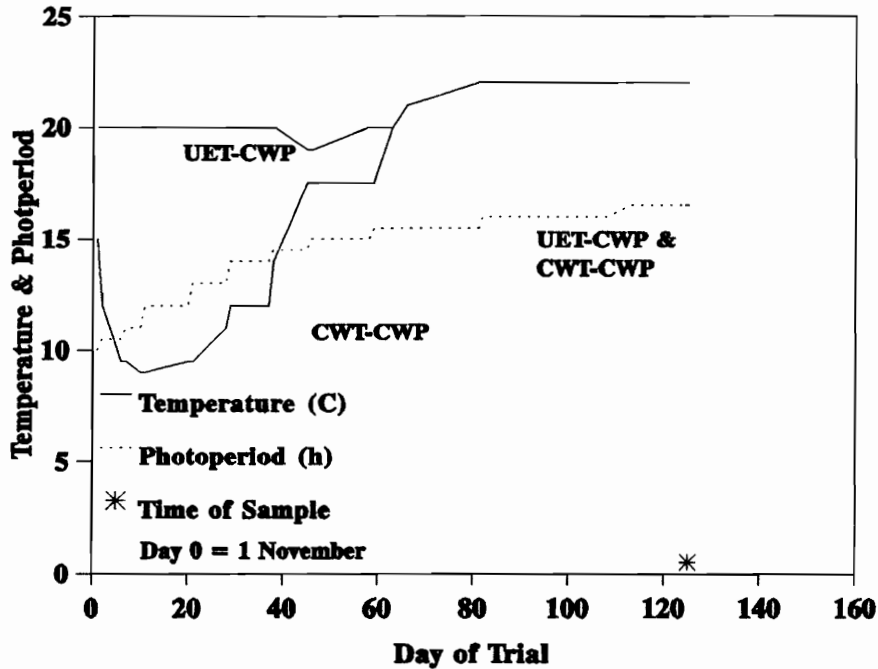
Figure 4.3. Approximate temperature and photoperiod regimes for captive fish held under a compressed photoperiod cycle, and exposed to either a compressed temperature cycle or constant temperature (Experiments CWT-CWP and UET-CWP).

cycle (Figure 4.3). Fish were collected and confined in late October.

4) CWT-CWP - captive fish held under compressed temperature and photoperiod cycles that started with a mid-winter temperature and photoperiod cycle (Figure 4.3). Fish were collected and confined in late October.

5) CNT-CNP - captive fish held under compressed natural temperature and photoperiod cycles (Figure 4.4). Fish were not gravid and had been held for several months in the oval raceway at 16 h light and 19 to 23 C. They

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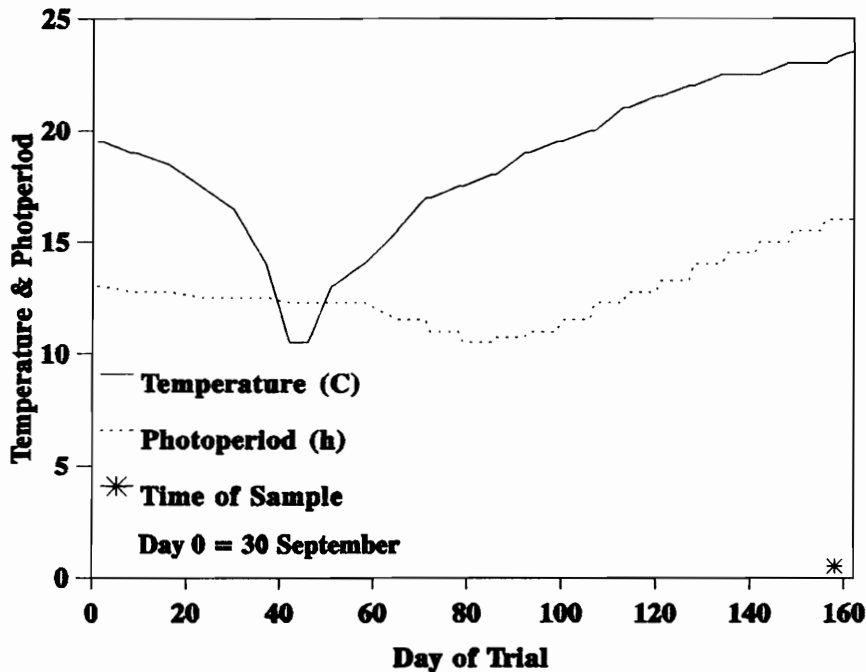
Figure 4.4. Approximate temperature and photoperiod regimes for captive fish held under compressed temperature and photoperiod cycles (Experiment CNT-CNP).

were moved to the twelve-tank system in late September.

Fish were acclimated to captivity prior to the beginning of experiments. They were considered acclimated if they readily fed on the prepared diet. The number of fish used in each experiment was dependent upon the number that could be captured at the time of the beginning of the experiment, and ranged from 30 to 128 (Table 4.1). Differences in gonadosomatic indices (GSI = (gonad weight/ body weight (including viscera) X 100) of males and females were used to assess the efficacy of each temperature and photoperiod combination to induce gonadal maturation. Nine GSI samples of fish in Experiment WF, and seven samples in Experiment NT-NP

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Table 4.1. Summary of experiments to evaluate the effects of photoperiod and temperature on the maturation of <u>Noturus</u> <u>insignis</u> gonads.

Experiment	Temperature[1]	Photoperiod[1]	Number of days	Beginning (ending) date	Tank type[1]	Number of fish[2]
WF	natural	natural	246	7 Nov (3 Jul)	NA[3]	NA
NT-NP	simulated natural	simulated natural	276	4 Oct (3 Jul)	7000 L oval	128
UET-CWP	uniform elevated	compressed winter	122	2 Nov (4 Mar)	530 L rectangular	30
CWT-CWP	compressed winter	compressed winter	122	2 Nov (4 Mar)	530 L rectangular	30
CNT-CNP	compressed natural	compressed natural	158	1 Oct (9 Mar)	110 L aquarium	72

[1]See text for more detailed descriptions of temperature-photoperiod regimes and tank types
[2]Number of fish stocked at beginning of each experiment
[3]Not applicable

were collected at regular intervals. GSI samples for all other experiments were collected only at the end of the experiments. Experiments were considered completed when spawning conditions were reached (photoperiod of 15 to 16 h light, and temperature of 22 to 23 C). Diameters of samples of oocytes from females were measured concurrent with the metrics to calculate GSI values for all experiments except CNT-CNP. In Experiment CNT-CNP diameters of oocytes from eight fish were measured, but the fish were not sacrificed to obtain information for GSI values, because they were used in a hormonal-implant experiment. GSI metrics and diameters of oocytes were collected for all other fish sampled at the end of Experiment CNT-CNP. Several fish from Experiments UET-CWP, CWT-CWP, and

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CNT-CNP were not sampled at the end of the experiments, but were maintained under spawning conditions (15 to 16 h light, and 22 to 23 C) for approximately three months. The GSI values of the fish were measured at that time.

Fish used in experiments were not collected at exactly the same time or from the same exact locations, consequently, GSI values were not compared with a general linear model. Instead, plots were constructed to visually assess differences among experiments, and the results of each experiment were compared statistically to that of a sample of wild fish (Experiment WF) using Student's t-test. The mean GSI values for the May sample of wild female margined madtoms was selected for comparisons with those of captive females because wild fish typically begin spawning in late May or early June at the sample sites. Thus, it was not necessary to ascertain whether wild females had spawned, and gonadal development would be representative of fish just prior to the spawning season (prespawning fish). The mean diameter of the vitellogenic (yolked) oocytes of females in each experiment was also compared to that of the May sample of wild females using Student's t-test. The early July sample of wild male margined madtoms was selected for comparisons with captive males because the date is near the middle of the spawning season for wild fish at the sample sites, and consequently, I expected a large proportion of wild males to be in spawning condition at that time.

Description of Gonads and Gametes

Ovaries and testes of margined madtoms preserved in 10 % formalin were exposed by removing a portion of the abdominal wall to determine the position and connections of the gonads. Motility of sperm of males used in maturation and spawning experiments was periodically checked by viewing samples of a macerated section of testis under a light microscope (250X to 400X), and then adding fresh water to activate the sperm. A morphological description of margined madtom spermatozoa was obtained by examining them under a light microscope (1000X) and reviewing photographs (400X). Mean head length, head width, and tail length of margined madtom spermatozoa were estimated from measurements obtained from the photographs. Duration of motility of sperm in fresh water and phosphate buffered saline was compared.

Ovaries of 20 fish were examined to determine the relationships between diameter and weight of vitellogenic oocytes, and weight of vitellogenic oocytes and ovary weight. Because vitellogenic oocytes within margined madtom ovaries are relatively uniform in size, ovaries were purposely selected that represented a wide range of oocyte diameters. To determine the relationship between oocyte diameter and weight, the mean weight and diameters of six oocytes from each ovary were measured. The natural logs (ln) of mean weights of oocytes were then regressed on mean oocyte diameters. The natural log of weight was used because weight of an oocyte was expected to vary exponentially with its diameter.

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To determine the relationship between weight of vitellogenic oocytes and total ovary weight, the ovaries from all 20 females were weighed. All vitellogenic oocytes were then removed, counted, and weighed. The total weight of oocytes from each ovary was divided by the total weight of the ovary (including the vitellogenic oocytes), and this value was multiplied by 100 to give the percentage of the ovary weight that was comprised of vitellogenic oocytes (i.e., the percentage ovary as maturing oocytes). The percentages of the ovaries as oocytes were then regressed on natural logs of the mean weights of oocytes. The natural log of mean weight was used because the weight of vitellogenic oocytes was expected to vary exponentially relative to the proportion of the ovary that was composed of vitellogenic oocytes.

Length-weight relationships were determined for male, gravid female, and nongravid female margined madtoms. A female was considered to be gravid if the GSI value was greater than 2.0. A length-fecundity relationship was developed for female margined madtoms by counting all vitellogenic oocytes from 20 females and then regressing the counts on total lengths of females.

Plasma Steroid Levels

Plasma samples were obtained from fish sacrificed to obtain GSI values in Experiments WF and NT-NP. All fish were anesthetized and then the tail was severed at the caudal peduncle. Heparinized Natelson blood collecting tubes (250 μl) were used to collect the blood. Blood was collected until no more could easily be

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obtained, and it was immediately transferred to a 1.0 mL microcentrifuge tube to which a drop of heparin (sodium salt, grade 1, from porcine intestinal mucosa, 1500 units per 1 mL water) had been added, and then placed on ice. Following collection of all blood samples on each sample date, the samples were centrifuged at 3000 rpm for 15 min. The plasma portion of the blood samples was then aspirated, placed into clean centrifuge tubes, labeled, and frozen until time of steroid analysis.

Standard radioimmunoassay (RIA) kits were used to measure concentrations of 17β-estradiol (females) or testosterone (males) in plasma samples (Diagnostic Products Corporation, Los Angeles). Plasma collected from several madtoms not used in experiments was pooled, and used to prepare standards and run validation tests. Activated charcoal and dextran were used to strip steroids from pooled plasma used to make standards (ratio of plasma to charcoal to dextran = 100:10:1).

Madtom plasma has not previously been used in RIAs, and consequently a test for parallelism using the standards, and samples of the pooled plasma that had not been stripped of steroids, was performed to validate its use in this study. Standards were prepared by spiking samples of the steroid-stripped plasma at levels of 0.0, 0.1, 0.5, 1.0, 2.0, and 5.0 ng/mL of testosterone, or with 0.0, 0.1, 1.0, and 2.0 ng/mL of 17β-estradiol. The unstripped plasma samples were spiked at the same levels as standards. Duplicate 100 μl samples were run for 17β-estradiol, and duplicate 50 μl samples were run for testosterone. These samples were incubated for the period of time recommended in the kits, and then steroid levels were measured.

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Fifty microliter (testosterone) and 100 μl (17β-estradiol) samples were also used to measure steroid levels of fish used in maturation experiments. The standards used were the same as those used in validation tests. All testosterone samples were analyzed in a single run. The 17β-estradiol samples were analyzed in two runs. In the second, an additional standard (4.0 ng/mL) was used. All samples were run in duplicate. For some samples, there was an insufficient amount of plasma for two complete samples. In those cases, one tube received the prescribed volume of plasma, and the amount of plasma that remained was measured and placed into the second tube. The volume of the tube was brought up to the prescribed level by adding steroid-free human plasma from the RIA kits. Concentrations of steroids were subsequently adjusted for samples that did not receive a full complement of madtom plasma. If count values obtained from duplicate samples varied more than 25 %, they were not used in analyses.

Total plasma volumes obtained from several fish were correlated with weight of fish to determine the volume of plasma that can be obtained from different sizes of madtoms. Steroid levels were plotted against GSI values to determine the relationship between size of gonad and plasma steroid concentration.

Multiple Injections of CPE

Fish in this experiment were obtained from a variety of sources, but all had been held in captivity at constant photoperiod (16 h) and temperature ($\approx$ 22 C) for

more than a year. Fish that had squarish heads and slightly developed head musculature were selected because they were known to be males. Three trials were conducted. In the first, four fish were injected with a total dosage of 1.63 μg of CPE over seven days. They were injected on days one, two, four, and seven. On day one they received 0.13 μg, and on days two, four, and seven they received 0.50 μg of CPE. Four control fish were not injected. On day eight the GSI of all fish was determined, and macerated sections of testes were examined for motile sperm.

In the second and third trials, fish were injected daily for 7 or 14 d. In the 7 d trial, four fish were injected with CPE and two control fish were injected with water. Those that received hormonal injections were given a total dosage of 2.5 μg of CPE. They received 0.25 μg on days one and two, 0.50 μg on day three, and 0.38 μg on days four through seven. The GSI of all fish was determined on day eight.

In the 14 d trial, three fish received daily injection of 0.5 μg of CPE, and three control fish received daily injections of water. The GSI of these fish was determined on day 15. In trials 2 and 3, the degree of vascularization of the testes, and relative abundance and motility of spermatozoa were determined.

To evaluate sperm motility, portions of homogenized testes were viewed under a compound light microscope (400X and 1000X), and then water or phosphate buffered saline was added to activate the sperm. The duration of motility of spermatozoa in each media was then recorded. Relative abundance of spermatozoa was determined concurrent with evaluations of sperm motility. Abundance was rated

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as either very low, low, medium, or high (under 1000X). A very low rating was given if repeated scanning of the slide revealed no or very few spermatozoa; low was few spermatozoa in each field as the slide was scanned; medium was a few to several dozen spermatozoa visible in each field; and a rating of high was given if dozens of spermatozoa were visible in most fields as the slide was scanned. The procedure was repeated two or three times for fish in which ratings of less than high were initially obtained.

Vascularization of testes was rated low, medium, or high. A low rating meant the testes were mostly white in color; medium indicated the testes had a pinkish tinge due to the presence of a few visible blood vessels; and high meant the testes were reddish in color due to prominent blood vessels.

Results

Temperature-Photoperiod Experiments

The trends of GSI values of male and female margined madtoms in Experiment NT-NP (Figures 4.5 and 4.6) was similar to that for fish collected from the wild (Figures 4.7 and 4.8). The mean GSI values for wild fish were highest in the 3 July sample (day 244) for males, and in the 24 May sample (day 205) for females. At the end of all experiments, most captive females had obtained GSI values similar to those of gravid wild fish collected during or just prior to the spawning season (GSI $\approx$ 7 or greater, Figures 4.5, 4.7, and 4.9). Within sample variation of GSI values for females was high only when spawning conditions were present. Within sample variation of GSI values was high for males in all experiments, including WF (Figures 4.6, 4.8, and 4.10).

The highest mean GSI for males at the end of experiments was that of wild fish (Table 4.2), but values obtained throughout the year were not statistically different (p-value = 0.31, Figure 4.8). Male GSI values for Experiments NT-NP, CNT-CNP and CWT-CWP (respective means and p-values; 0.49 and 0.22, 0.58 and 0.85, and 0.52 and 0.33) were similar to that obtained for wild fish during the spawning season (mean GSI = 0.60), but those for Experiment UET-CWP were lower (mean = 0.38, p-value = 0.01).

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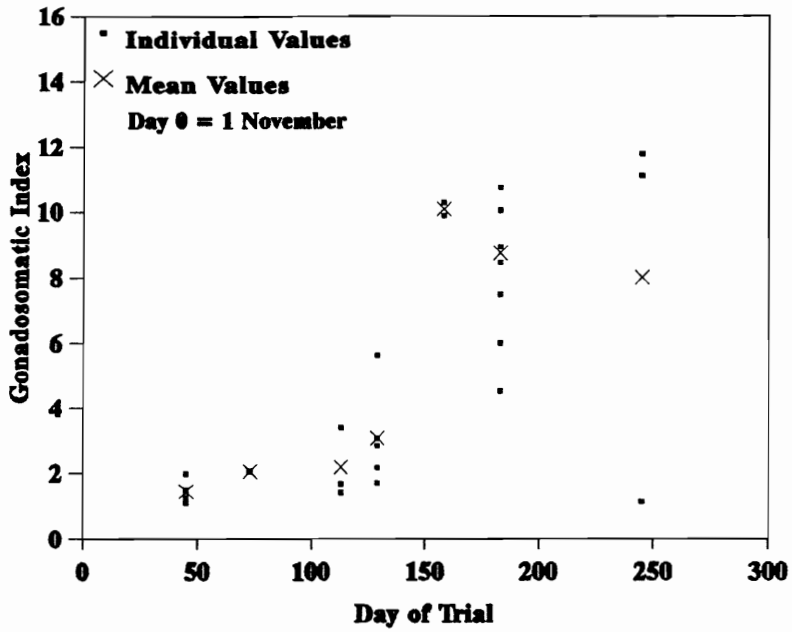
Figure 4.5. Gonadosomatic indices of captive female <u>Noturus</u> <u>insignis</u> held under simulated natural temperature and photoperiod regimes (Experiment NT-NP).
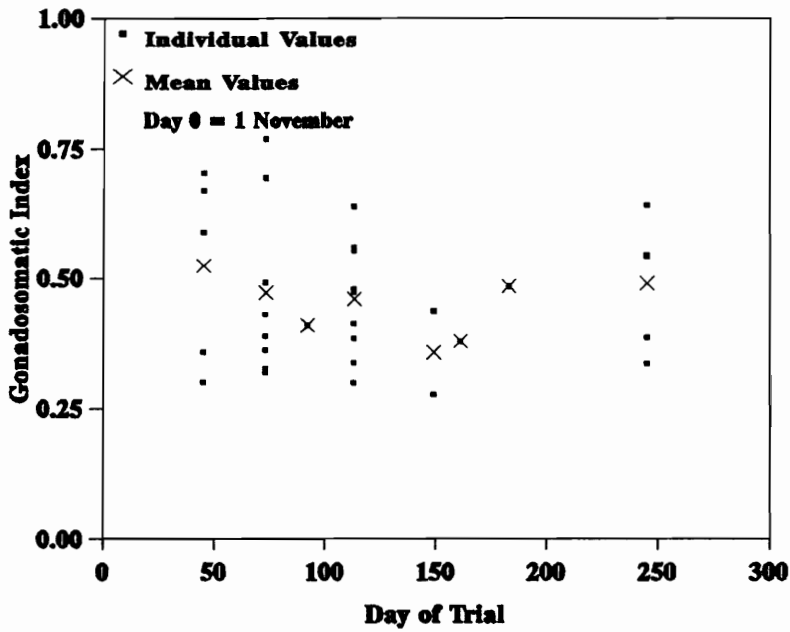
Figure 4.6. Gonadosomatic indices of captive male <u>Noturus</u> <u>insignis</u> held under simulated natural temperature and photoperiod regimes (Experiment NT-NP).

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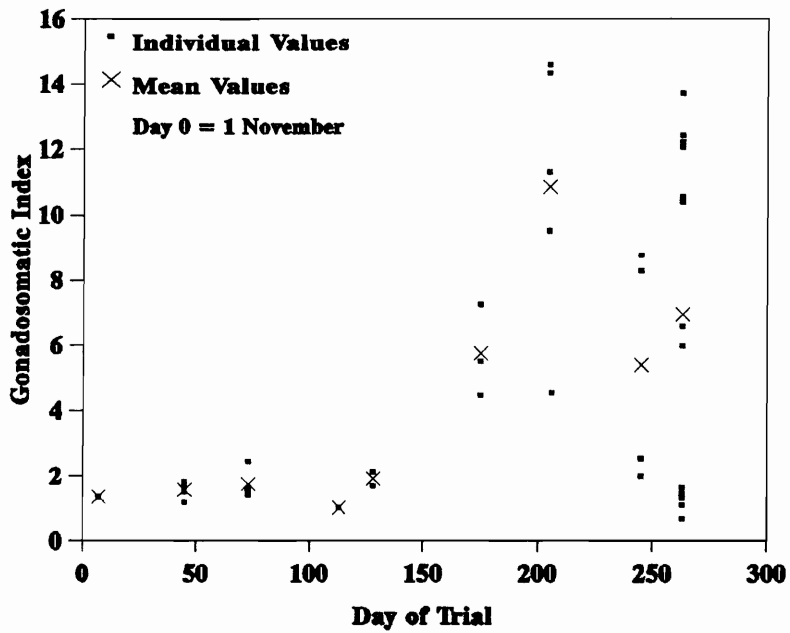
Figure 4.7. Gonadosomatic indices of female <u>Noturus</u> <u>insignis</u> collected from the wild.
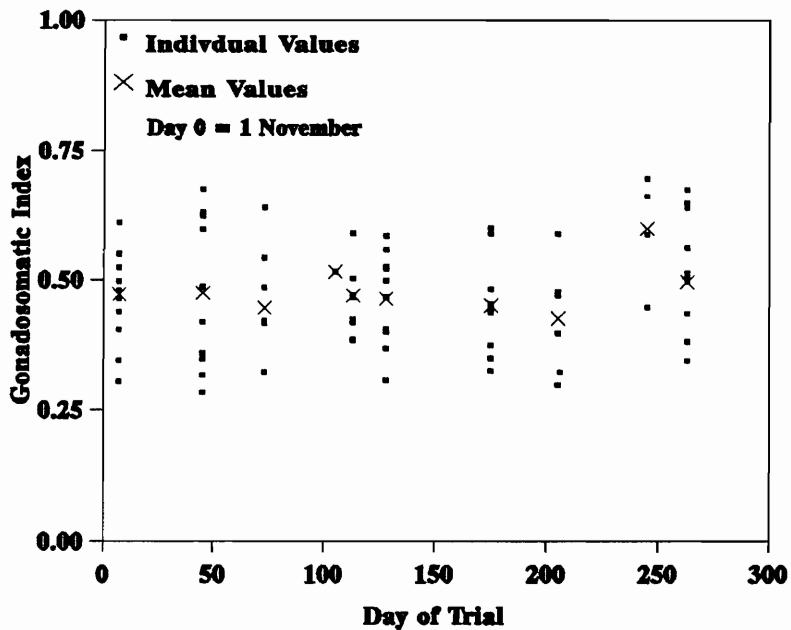
Figure 4.8. Gonadosomatic indices of male <u>Noturus</u> <u>insignis</u> collected from the wild.
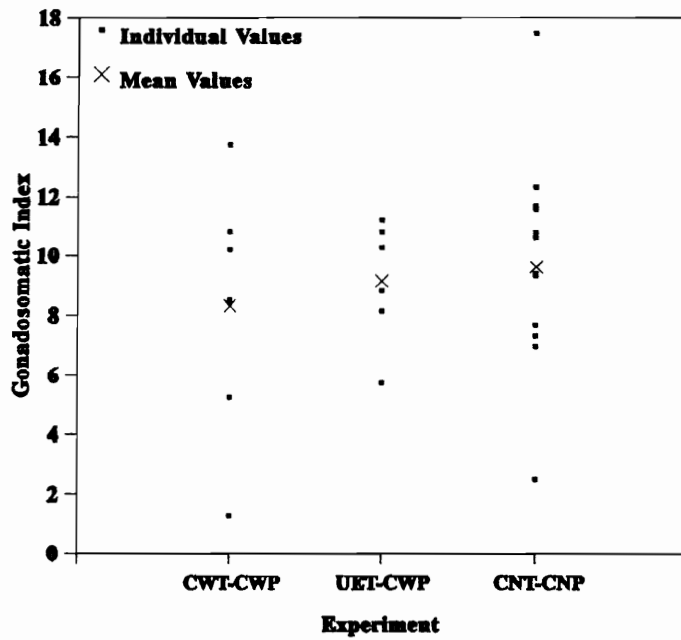
Figure 4.9. Gonadosomatic indices of captive female <u>Noturus</u> <u>insignis</u> held under three different temperature and photoperiod regimes.
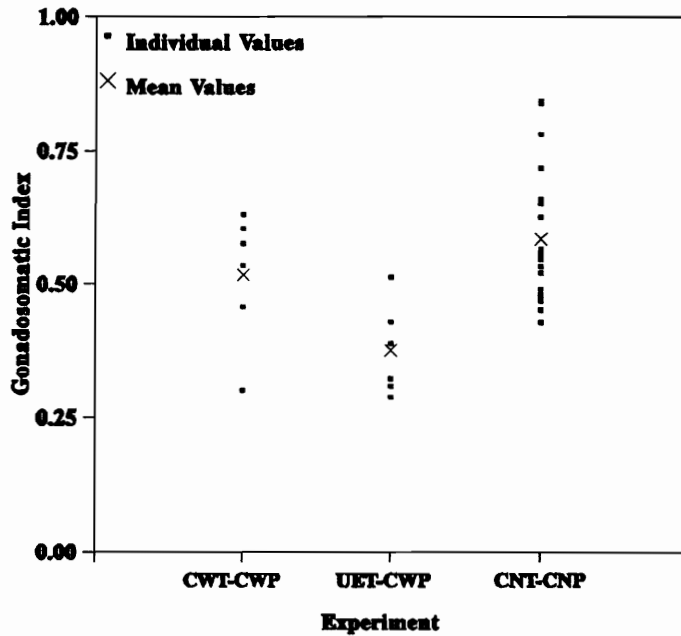
Figure 4.10. Gonadosomatic indices of captive male <u>Noturus</u> <u>insignis</u> held under three different temperature and photoperiod regimes.

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Table 4.2. Description and results of experiments to manipulate maturation of the gonads of male <u>Noturus</u> <u>insignis</u>.

Trial (number of males)	Tank type	Trial length (d)	Days[1] from normal season	Mean[2] GSI (p-value)
WF (4)	NA[4]	Nov to Jul (231)	0	0.60[3] (NA)
CNT-CNP (19)	110 L aquaria	Oct to Mar (173)	-83	0.58 (0.85)
CWT-CWP (6)	500 L rectangular	Nov to Mar (156)	-87	0.52 (0.33)
NT-NP (5)	7000 L oval	Oct to Jul (270)	-33	0.49[3] (0.22)
UET-CWP (6)	500 L rectangular	Nov to Mar (156)	-87	0.38 (0.01)

[1]Assumes spawning season for wild fish begins 1 June; minus is used to indicate days before normal season
[2]Probability that GSI value differs from that of wild fish collected in July
[3]July sample only
[4]Not applicable

Wild females also had the highest GSI value of all treatments, but the smallest mean diameter of oocytes (3.08 mm). Most captive females in all experiments had large oocytes (range of means 3.34 to 3.44 mm), and the mean values of fish in all experiments, other than CNT-CNP (p-value = 0.08), were statistically larger than those of wild fish. Mean GSI values of captive fish (range of means = 8.31 to 9.61) were similar (range of p-values = 0.29 to 0.53) to the mean GSI value of wild fish collected just prior to the spawning season (10.9, Table 4.3). Based on external characteristics, most females were gravid at the end of maturation experiments,

Table 4.3. Description and results of trials to manipulate maturation of the gonads of female <u>Noturus</u> <u>insignis</u>.

Trial (number of females)	Tank type	Trial length (d)	Days[1] from normal season	Mean GSI (p-value)[2]	Mean oocyte diameter (mm) (p-value)[2]
WF (5)	NA[4]	Nov to Jul (231)	0	10.86[3] (NA)	3.08[3] (NA)
CNT-CNP (13)	110 L aquaria	Oct to Mar (173)	-83	9.61 (0.53)	3.31 (0.08)
UET-CWP (6)	500 L rectangular	Nov to Mar (156)	-87	9.16 (0.39)	3.44 (0.01)
NT-NP (8)	7000 L oval	Oct to Jul (270)	-33	8.74[3] (0.29)	3.37[3] (0.02)
CWT-CWP (7)	500 L rectangular	Nov to Mar (156)	-87	8.31 (0.31)	3.38 (0.03)

[1]Assumes a 15 June to 15 August spawning season for wild fish; minus is used to indicate days before normal season
[2]Probability that value differs from that of wild fish collected in May
[3]May sample only
[4]Not applicable

however, no fish held under spawning conditions for an additional three months were gravid at the end of the time period. The mean GSI value of females at that time was 1.29 ± 0.21 (N = 15), and the mean GSI value of males was 0.36 ± 0.11 (N = 10).

Description of Gonads and Gametes

The ovaries of the margined madtom consist of paired cylindrical organs located below the kidney and attached to the dorsal mesentery in the coelomic cavity. The halves connect at the oviduct and oocytes exit the body via the genital pore which is located between the anus and the urinary opening. The testes consist of paired elongate masses of villiform lobes. The villiform lobes connect to a central sperm duct that runs parallel to the long axis of the body. The two sperm ducts join to form a common sperm duct that empties in the urinary, posterior to the bladder. Spermatozoa exit the body via the urogenital pore. The villiform lobes of the anterior portion of the testis are smaller than those of the posterior portion.

Motile sperm were observed only once in a sample of milt from macerated testes to which phosphate buffered saline had been added. The time of motility of the sperm was < 20 sec. No motile sperm were observed when fresh water was used to activate them, and destruction of sperm in fresh water occurred within seconds. In general, number of sperm observed in all studies was very low. The heads of the spermatozoa are slightly ovate ($4.3 \pm 0.2 \, \mu$m long and $3.6 \pm 0.2 \, \mu$m wide). The tails are centrally attached to the head. The lengths of the tails of spermatozoa from margined madtoms could not be determined from photographs because none of them contained a complete tail, but they are $> 112.5 \, \mu$m long. A pronounced, collar-like, midpiece encircles the posterior portion of the head and anterior portion of the tail.

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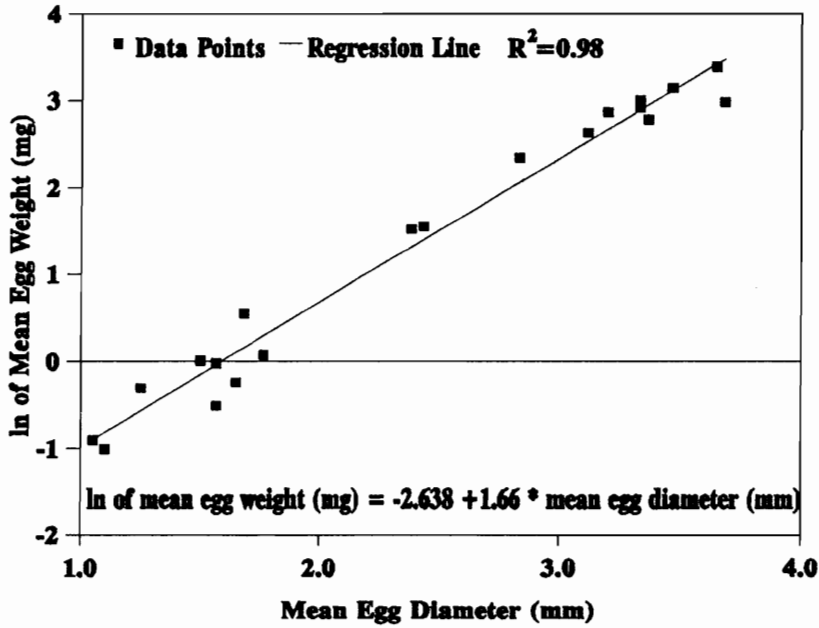
Figure 4.11. Relationship between mean diameter and ln of weight of oocytes of <u>Noturus</u> <u>insignis</u>.

The regression coefficients were very high for the regression of the natural log of the mean egg weight on mean egg diameter ($R^2 = 0.98$), and for the regression of percent of ovary as eggs on the natural long of the mean egg weight ($R^2 = 0.94$, Figures 4.11 and 4.12). The regression of egg weight on diameter includes ova that ranged from ≈ 1 to 4 mm in diameter (Figure 4.11). The percent of the ovary as eggs increased from ≈ 20 % in ovaries that have oocytes ≈ 1 mm in diameter to ≈ 80 % in ovaries with very large oocytes (Figure 4.12).

The length-weight relationship for gravid females was slightly greater than that for males or nongravid females, but plots of the relationships show that the difference is small (Figure 4.13). Consequently, a single length-weight equation was developed

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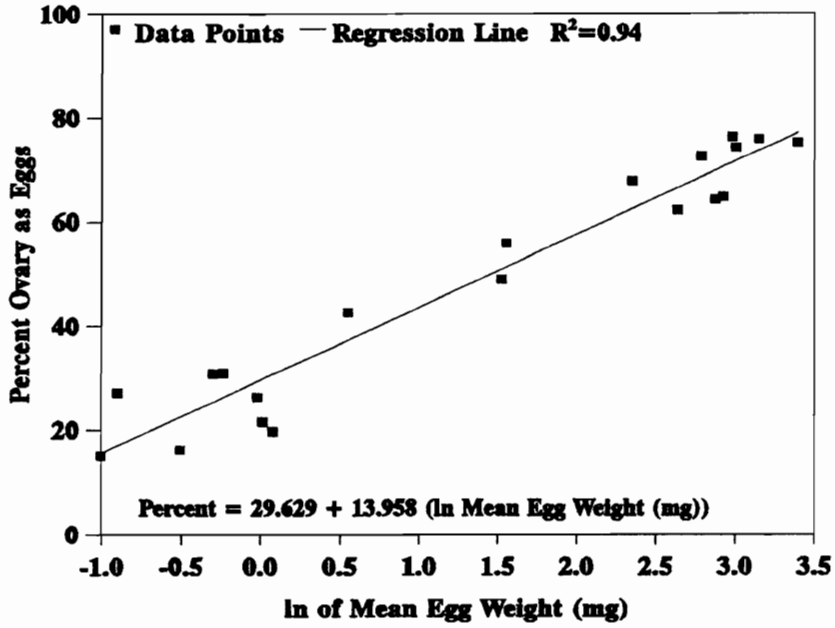
Figure 4.12. Relationship between ln of mean weight of oocytes and percent weight of ovary as oocytes for <u>Noturus insignis</u>.
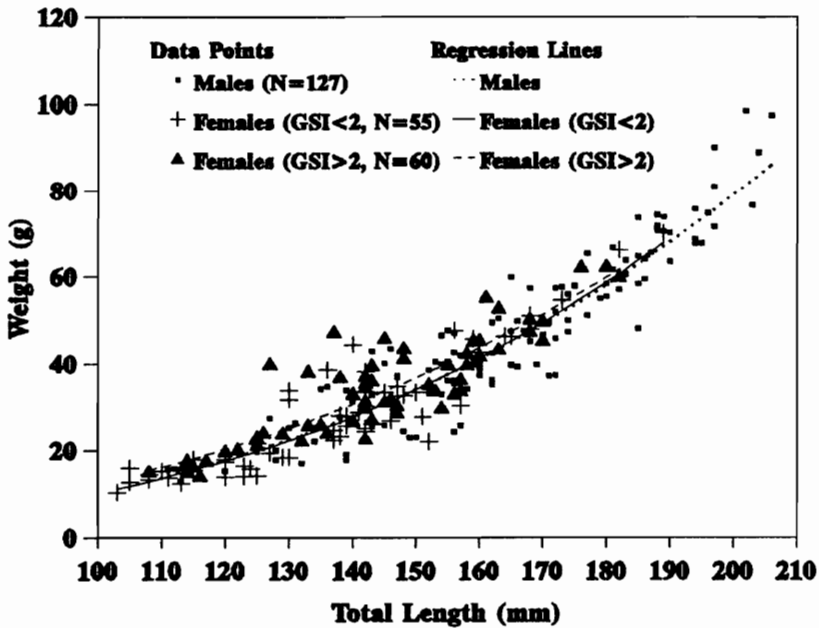
Figure 4.13. Length-weight relationships for <u>Noturus insignis</u>.

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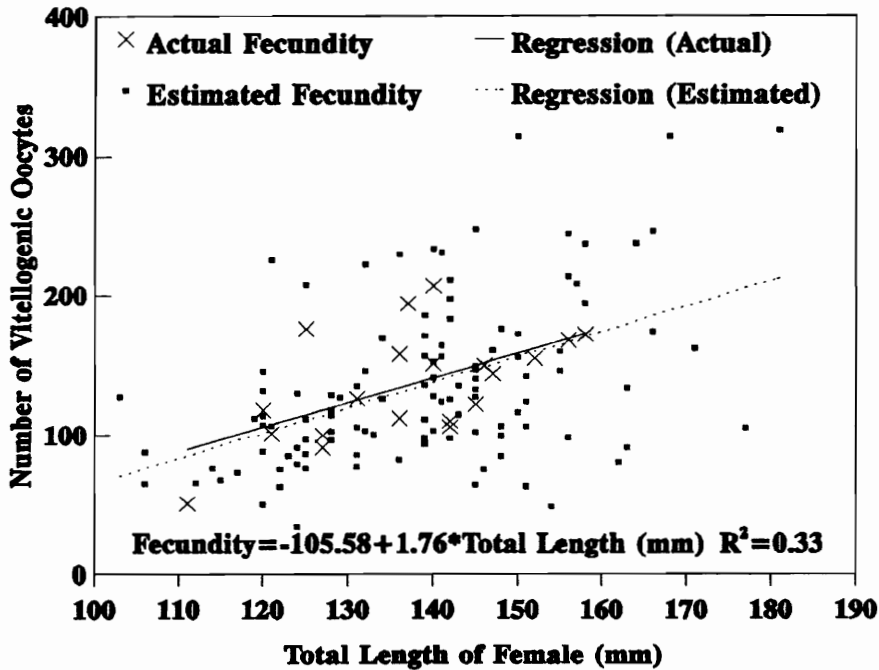
Figure 4.14. Length-fecundity relationship for female <u>Noturus</u> <u>insignis</u>. Regression equation is for actual data points.

for all margined madtoms: ln weight (g) = -10.77 +2.86 * ln total length (mm) (N = 242, R^2 = 0.87). The GSI values for females considered to be gravid ranged from 2.1 to 7.4 (mean = 3.6 ± 1.1), and for nongravid females they ranged from 0.1 to 2.0 (mean = 0.8 ± 0.5).

Number of vitellogenic oocytes was moderately and positively correlated with total length (N = 20, R^2 = 0.33, Figure 4.14). The absolute fecundity of 20 females ranged from 42 to 207 vitellogenic oocytes and averaged 129.3 ± 41.9. The absolute fecundity of 112 female margined madtoms estimated from the equation; ovary weight * proportion of the ovary as eggs / mean weight of the ova, where mean

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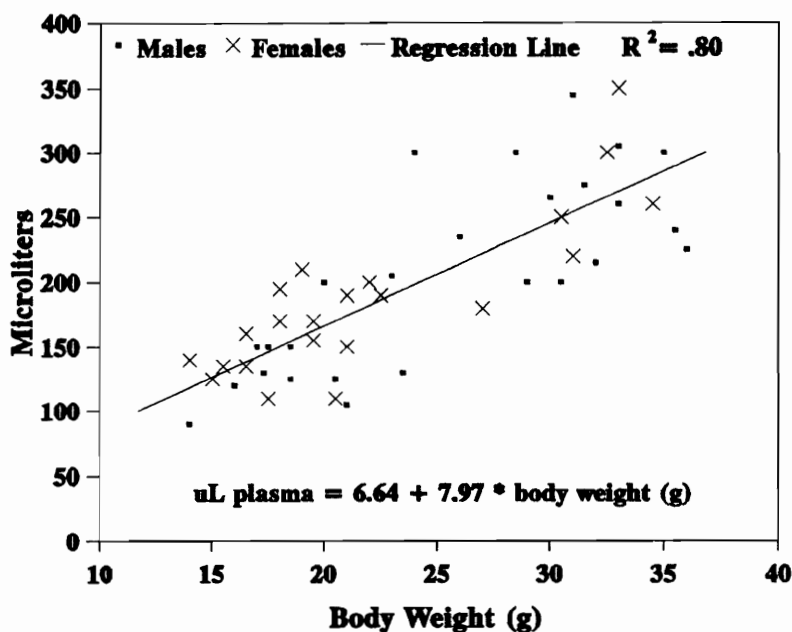
Figure 4.15. Available plasma volumes from <u>Noturus</u> <u>insignis</u> of different sizes.

weight of ova was estimated from mean diameter, ranged from 24 to 318 and averaged 133.4 ± 59.2 ova.

Plasma Steroid Levels

Sufficient plasma volumes could be obtained from most fish to run duplicated samples (Figure 4.15). The relationship between body weight and plasma volume obtained was μL of plasma = 6.64 + 7.97 * body weight (g) (N = 48, R^2 = 0.80). Thus, 100 μL of plasma can be obtained from a fish that weighs approximately 11.7 g.

Assay binding in the first validation test was 37 % for testosterone and 52 % for 17β-estradiol. The slope and correlation coefficients were within acceptable levels

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in both runs. In the testosterone validation test they were 1.11 and 0.99, and for the 17β-estradiol tests they were 1.17 and 0.98.

Assay binding was 33.0 % in the testosterone run, and 26.0 and 50.4 % in the 17β-estradiol runs. Seventeen plasma samples of wild fish and 10 samples of captive fish were analyzed for testosterone. Twenty-two plasma samples of wild fish and 17 samples of captive fish were analyzed for 17β-estradiol. Thirty-two of the 17β-estradiol samples were analyzed in the first run, and seven were analyzed in the second run.

Estimated plasma concentrations of several samples of testosterone and 17β-estradiol were greater than those of the highest standards; the maximums were 4 ng/mL for 17β-estradiol, and 5 ng/mL for testosterone (Figures 4.16 and 4.17). Steroid levels for wild fish began to rise in the spring and peaked during or just before the spawning season. Steroid levels for captive fish were similar to those of wild fish, except that they were more variable. The highest levels of 17β-estradiol were associated with intermediate GSI values of females, but there was no relationship between testosterone and GSI values for males (Figures 4.18 and 4.19)

Multiple Injections of CPE

Multiple injections of CPE resulted in slight increases of GSI values for hormonally-injected fish, but sperm number in these fish was lower than that for control fish (Figure 4.20 and Table 4.4). Motile sperm were not observed in any

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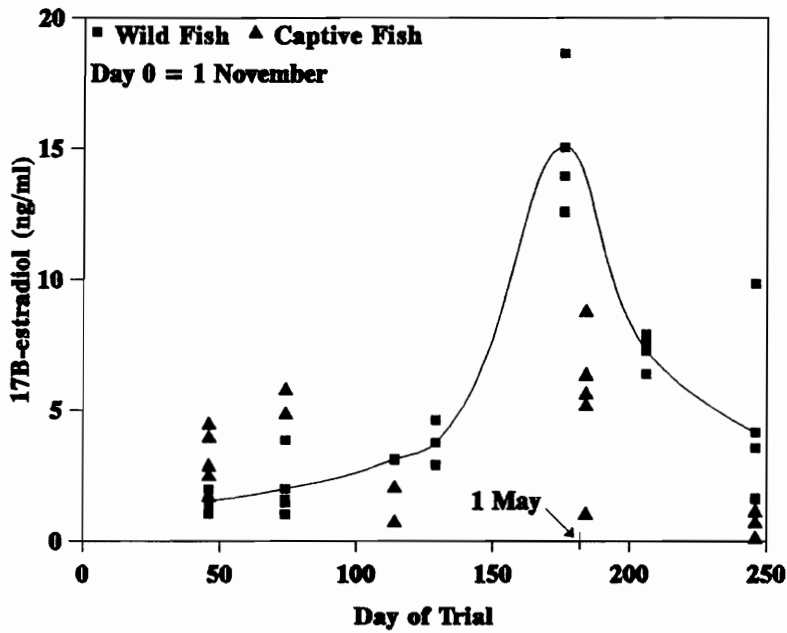
Figure 4.16. Seasonal changes of 17β-estradiol in the plasma of <u>Noturus</u> <u>insignis</u> females. Line fit to mean values of samples for wild fish.
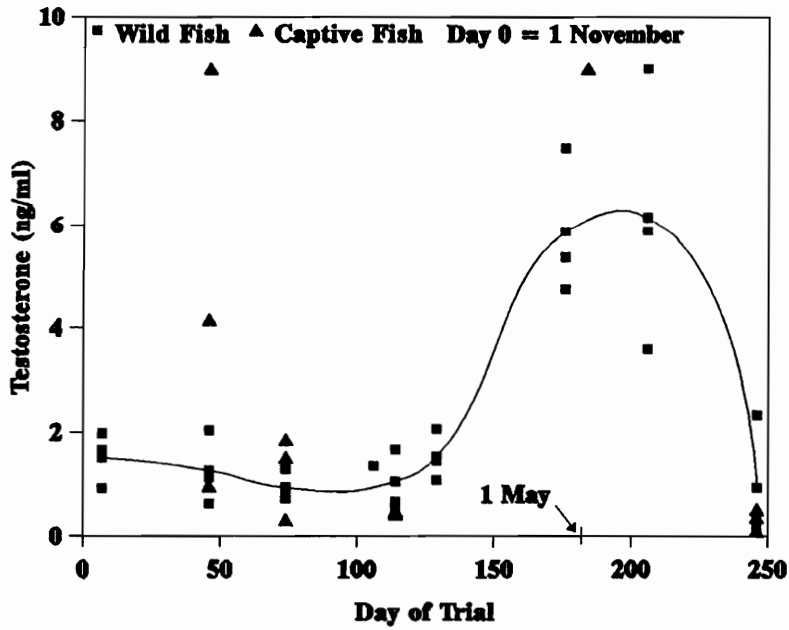
Figure 4.17. Seasonal changes of testosterone in the plasma of <u>Noturus</u> <u>insignis</u> males. Line fit to mean values of samples for wild fish.

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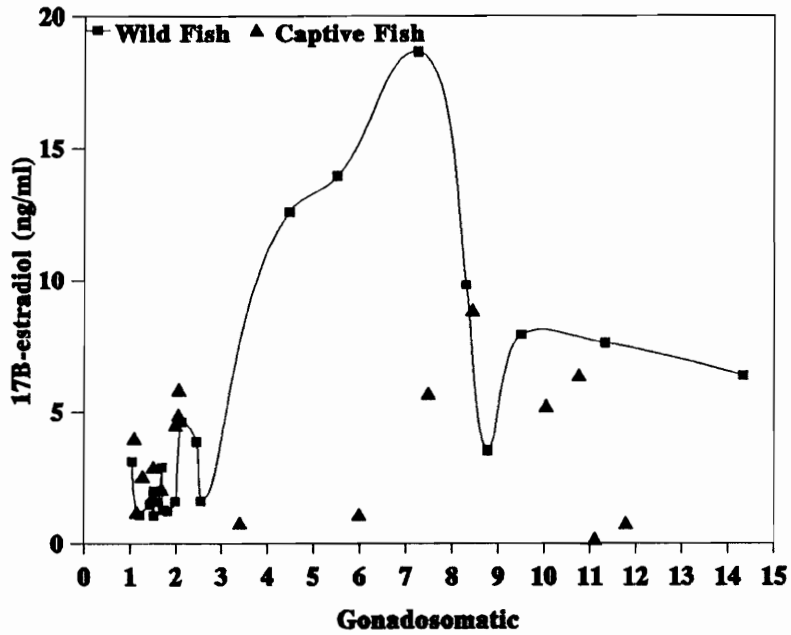
Figure 4.18. Relationship between 17β-estradiol plasma levels and gonadosomatic index of female <u>Noturus</u> <u>insignis</u>. Line fit to values for wild fish.
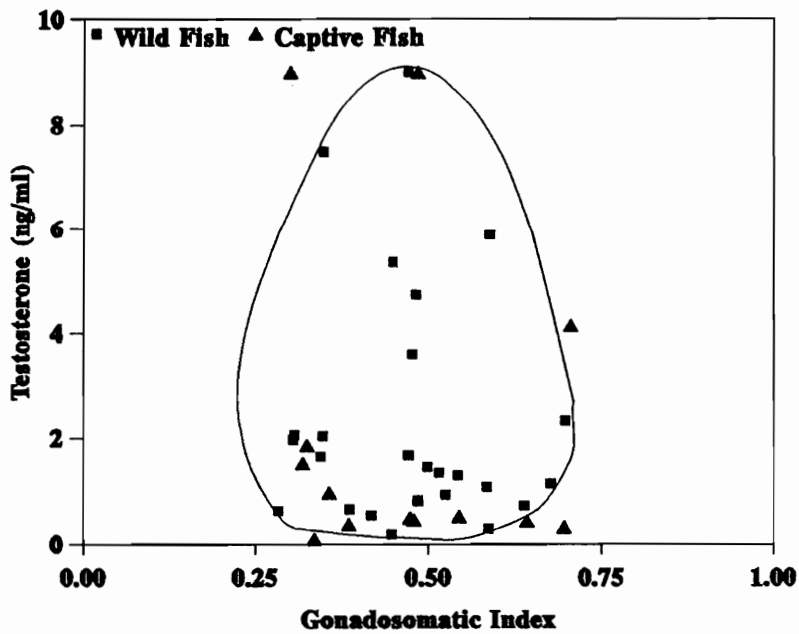
Figure 4.19. Relationship between plasma testosterone levels and gonadosomatic index of <u>Noturus</u> <u>insignis</u> males. Line encompasses values for wild fish.
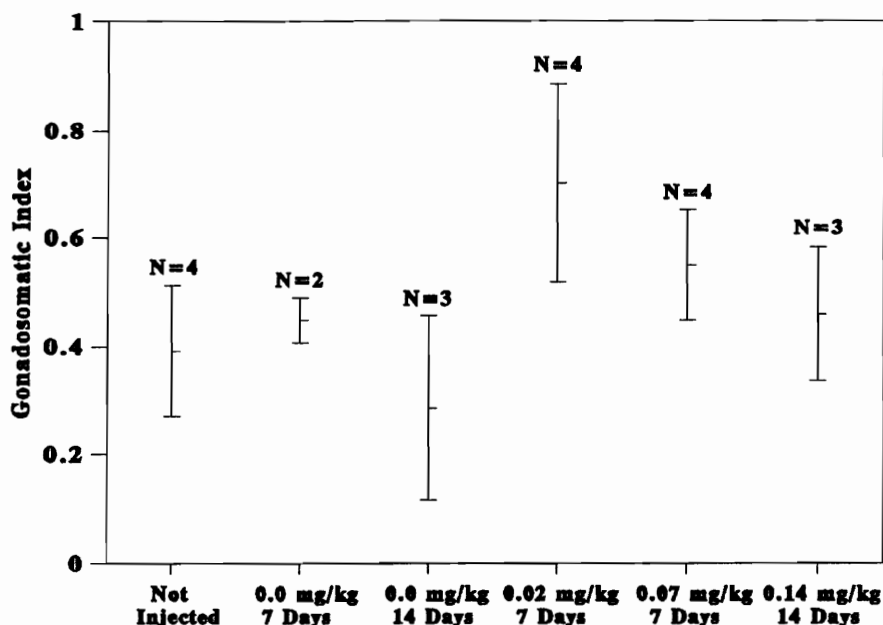
Figure 4.20. Comparison of gonadosomatic index values of <u>Noturus</u> <u>insignis</u> males that received multiple injections of CPE, and those that did not. Tic marks on bars are mean ± two standard deviations.

Table 4.4. Comparison of sperm number and vascularization of testes of <u>Noturus</u> <u>insignis</u> that received multiple injections of carp pituitary extract, and those that did not.

	Injection dosage		
	0 mg/kg/d 7 or 14 days	0.05 to 0.12 mg/kg/d - 7 days	0.10 to 0.7 mg/kg/d - 14 days
Number of sperm			
very low	0	1	3
low	0	1	1
medium	0	1	0
high	5	1	0
Vascularization of testes			
low	2	1	0
medium	1	1	1
high	0	2	3

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hormonally-injected fish, and in only one control. Testes of fish that received hormonal injections were notably more vascularized than fish that did not receive hormonal injections.

Discussion

This discussion is divided into five sections. The first two sections focus on the biological characteristics of the gonads of Noturus spp., the third on effects of environmental manipulation on gonadal manipulation, the fourth on seasonal changes in GSI values and plasma hormonal levels, and the fifth on strategies to induce and maintain gonadal maturation of rare madtoms. Information on the biology of species is generalized to the generic level where appropriate. The discussion is couched in a context for developing techniques to induce and maintain spawning condition of rare madtoms as part of a controlled propagation program.

Structure and Function of the Testes

The testes of the margined madtom conform to the general morphological form of other ictalurid testes. They contrast with the paired, smooth, cylindrical organs of most other teleosts (Grizzle 1985). The testes of ictalurids are paired elongate masses of villiform lobes. Small anastomosing sperminiferous tubules connect the villiform lobes to sperm ducts that are oriented parallel to the proximal testis and joined in the vicinity of the urinary bladder, to form a common sperm duct that empties into the urinary duct, posterior to the bladder (Burke and Leatherland 1984, Grizzle 1985). Spermatozoa exit the body via the urogenital pore.

Lobules of the anterior and posterior portion of ictalurid testes differ in histology and function (Sneed and Clemens 1963, Grizzle and Rogers 1976). Those

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of the anterior three quarters of the testis produce spermatozoa, and their lumina (seminiferous tubules) are lined with spermatogenic cells (cuboidal cells in various stages of division and Sertoli cells (Sneed and Clemens 1963, Burke and Leatherland 1984, Grizzle 1985, Rosenblum et al. 1987). It is very likely that the Leydig cells, based on their content, are a source of testicular androgens, as is the case for most teleosts (Grizzle 1981, Burke and Leatherland 1984). Sertoli cells also produce or modify androgens in some teleosts, but their role in androgen production of ictalurids is not known (Grizzle 1981, Nagahama 1983).

The lumina of lobules in the posterior quarter are lined with columnar epithelial cells and are devoid of germ cells (Sneed and Clemens 1963, Rosenblum et al. 1987). Burke and Leatherland (1984) and Rosenblum et al. (1987) observed elongation of these cells during the spawning season in the brown bullhead, Ictalurus nebulosus. Rosenblum et al. (1987) concluded that the posterior region of the testis is specialized for fluid secretion, but Sneed and Clemens (1963) have shown that secretions from the posterior region are not critical for sperm viability or successful fertilization of ova of channel catfish. Hence, the specific function of the posterior region or gland is unknown. The histology of madtom testes has not been studied.

Lobules of the anterior and posterior region of the white catfish, Amerius catus, are distinctly different that those of the blue catfish, Ictalurus furcatus, channel catfish, brown bullhead, the black bullhead, Amerius melas, and the flathead catfish, Pylodictis olivaris, (Sneed and Clemens 1963). In the latter, the posterior lobules are

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smaller than anterior lobules when the testis contains mature spermatozoa. In the white catfish, the posterior lobules are longer than those of the anterior region, and Sneed and Clemens (1963) found the epithelial cells of the posterior region to be similar, but finer in structure, than those of the channel catfish. More importantly, no spermatozoa (only spermatids) were observed in smear or stained sections, even during the spawning season, whereas spermatozoa are common in testes of several other ictalurids year-round (Sneed and Clemens 1963, Jaspers et al. 1978, Rosenblum et al. 1987). Sneed and Clemens (1963) postulated that the final stage of maturation of spermatozoa in the testes (conversion of spermatids to spermatozoa) occurs quickly in the white catfish and is initiated by an environmental trigger. Burke and Leatherland (1984) reported that spermatid production in brown bullheads occurs only during a three week period (the breeding season).

The testes of the margined madtom are similar in gross morphology to those of the white catfish, and few to no spermatozoa are present in smears of macerated testes, even during the spawning season. This contrasts with the findings of Trauth et al. (1981) who reported spermiogenesis in the frecklebelly madtom, Noturus munitus, from June to July, accompanied by abundant spermatozoa in cysts of the anterior lobe in June. They did not report if motility could be induced by the addition of water to the spermatozoa. When spermatozoa were present in smears of testes of the margined madtom, addition of water did not result in motility of spermatozoa as is typical of other species. There apparently are at least two

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functional types of ictalurid testes. Those that contain spermatozoa year-round, and those that seem to produce functional numbers of spermatozoa just prior to spawning. An understanding of the spermiation process in ictalurids that do not harbor spermatozoa in lumina of the testis year-round is highly desirable, from the perspective of developing controlled propagation techniques for these species.

The testes of catfishes are relatively small. The GSI even during the spawning season is typically less than 0.9 for the channel catfish and the brown bullhead (Guest et al. 1976, Jaspers et al. 1978, Jearld and Brown 1971, Burke and Leatherland 1984, Rosenblum et al. 1987). Mayden and Burr (1981) found maximum GSI values of approximately 0.7 for the slender madtom, Noturus exilis, and I have found maximum values of 0.84 for captive, and 0.70 for wild margined madtoms. The small size and morphology of the testes precludes stripping of milt from males. Consequently, strip spawning necessitates sacrifice of the male.

Structure and Function of the Ovaries

The ovaries of the margined madtom are similar to those of other ictalurids (Clemens and Sneed 1963). As oocytes mature they compose a larger proportion of the ovaries. The strong correlations between percent of ovary as oocytes and weight of oocytes, and between oocyte diameter and weight, allows prediction of fecundity from mean oocyte diameter and total weight of the ovaries. Fecundity estimates obtained by using these predictors compared favorably with actual counts from 20

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females of similar lengths (see Figure 4.11). Fecundity was positively, but only moderately correlated with length (R^2 for actual fecundities $= 0.33$ and for estimated fecundities 0.25). This implies that environmental conditions, or individual condition of females, have a relatively strong effect on ovarian development among fish. The absolute fecundity of the margined madtom is high compared to other madtoms, but fecundity of madtoms in general is low when compared to other ictalurids (generally less than 200 oocytes per female, Table 4.5). Observed clutch sizes were similar to observed and estimated fecundities indicating that the ovaries of the margined madtom very likely are synchronous and only one clutch of ova are produced per year. Margined madtoms will resorb their oocytes several weeks after spawning condition are reached. The resorption of oocytes in other species of madtoms held in captivity has been observed by others (Bowen 1980, Mayden and Walsh 1984), and based on the results of tank-spawning trials, oocyte resorption will occur if spawning is not hormonally induced. Consequently, it is important to monitor progress of individual females and induce spawning when the ovaries have matured (see Chapter 5 for discussion of tank-spawning trials and methods to induce spawning).

Environmental Control of Maturation

Captive margined madtom females can mature out-of-season under a variety of photoperiod and temperature regimes. Bowen (1980) brought juvenile brindled

Table 4.5. Factors associated with gonadal maturation and reproduction of ictalurids.

Source	Species	Diameter of yolked ovarian oocytes-mm	Diameter of fertilized egg's chorion (yolk)-mm	Absolute fecundity (clutch size)	Female (male) GSI	Age or size at maturity
Ryder 1883	Ameiurus catus	1.6[1]	3.2[1] (1.6[1])	(2000[1])	---	---
Fowler 1917	Ameiurus catus	---	---	(1400-1500[1])	---	---
Menzel 1943	Ameiurus catus	4.0-4.5	---	3200-3500	---	18-20 cm
Dennison 1970	Ameiurus melas	---	---	3892[2]	---	---
Campbell and Branson 1978	Ameiurus melas	0.5[2]	---	2552-3372	12.1[2] (2.1[2])	---
Forney 1955	Ameiurus melas	---	---	3000-4000[1]	---	---
Fowler 1917	Ameiurus melas	---	---	(200[1])	---	---
Wallace 1972	Ameiurus natalis	---	3[1]	(200[1])	---	---
Fowler 1917	Ameiurus natalis	---	---	(300,700)	---	---
Blumer 1985	Ameiurus nebulosus	---	---	1500-2650[1] (1163[2])	---	---
Rosenblum et al. 1987	Ameiurus nebulosus	---	---	---	7.0[2] (0.46[2])	---
Burke et al. 1984	Ameiurus nebulosus	---	---	---	8.1[2] (0.22[2])	---
Dean 1891	Ameiurus nebulosus	---	3.2[1]	---	---	---
Wang and Kernehan 1979	Ameiurus nebulosus	---	3.0[1] (2.0[1])	---	---	---
Smith and Harron 1904	Ameiurus nebulosus	---	3.2[1]	(2000[1])	---	---
Fowler 1917	Ameiurus nebulosus	---	---	(50-500[1])	---	---

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Table 4.5 continued. Factors associated with gonadal maturation and reproduction of ictalurids.

Source	Species	Diameter of yolked ovarian oocytes-mm	Diameter of fertilized egg's chorion (yolk)-mm	Absolute fecundity (clutch size)	Female (male) GSI	Age or size at maturity
Breeder 1935	Ameiurus nebulosus	---	3.0[1]	(500-2000[1])	---	---
Hildebrand and Towers 1929	Ameiurus nebulosus	---	---	30,000[1]	---	---
Titcomb 1920	Ameiurus nebulosus	---	---	2188	---	---
Muncy 1959	Ictalurus punctatus	---	---	2628-9721	15[2]	---
Jearld and Brown 1971	Ictalurus punctatus	---	---	1052-64629	7.1[2] (1.0[2])	---
Harlan and Speaker 1956	Ictalurus punctatus	---	3.5[2]	---	---	---
Saskena et al. 1961	Ictalurus punctatus	---	(3.2[2])	---	---	---
Canfield 1947	Ictalurus punctatus	---	---	2000-8000	---	---
Mobley 1931	Ictalurus punctatus	---	---	10000-20000	---	---
Brauhn and McCraren 1975	Ictalurus punctatus	---	---	---	0.6-15.6, 10.5[2]	---
MacKenzie et al. 1989	Ictalurus punctatus	---	---	---	2.7-10.9	---
Clemens and Sneed 1957	Ictalurus punctatus	---	---	(1360/kg[1])	---	---
Menzel 1943	Ictalurus punctatus	3.5-4.0	---	42000-106000	---	23-25 cm
Brauhn 1971	Ictalurus punctatus	---	---	(2817-17297)	---	---
Shira 1917a	Ictalurus punctatus	---	---	(6000[1])	---	---
Shira 1917b	Ictalurus punctatus	---	3.5	---	---	---
Markmann and Doroshov 1983	Ictalurus punctatus	0.9-3.5, 2.3-3.0[2]	---	---	12.0	---
Burr and Mayden 1984	Noturus albater	---	---	(60-85, 71[2])	---	---

Table 4.5 continued. Factors associated with gonadal maturation and reproduction of ictalurids.

Source	Species	Diameter of yolked ovarian oocytes-mm	Diameter of fertilized egg's chorion (yolk)-mm	Absolute fecundity (clutch size)	Female (male) GSI	Age or size at maturity
Mayden et al. 1980	Noturus albater	2.1-2.3, 2.2²	3.5-4.0, 3.7² (2.9-3.0, 3.0²)	45-116, 90² (42)	---	1 yr
Dinkins 1982	Noturus baileyi	0.9-1.0, 1.0²	2.6-3.0, 2.8² (0.8-1.0, 0.9²)	55 (30-42, 36²)	---	1-2 yr
Burr and Dimmick 1981	Noturus elegans	---	4.1-4.4, 4.2² (2.9-3.0, 2.9²)	19-42, 31² (25-30)	---	---
Starnes and Starnes 1985	Noturus eleutherus	---	---	55-91 (70)	---	---
Burr and Mayden 1984	Noturus exilis	---	(3.5²)	(26-124, 72²)	---	---
Mayden and Burr 1981	Noturus exilis	1.5-3.4, 2.5²	3.9-4.5, 4.1² (3.2-3.6, 3.4²)	26-150, 84² (27-74, 51²)	21.1², 30.9³ (0.71³)	2 yr
Burr and Mayden 1984	Noturus flavater	1.3-3.2, 2.0²	4.6-5.0, 4.8² (3.4-3.7, 3.6²)	93-340, 240² (200-324¹)	4.9-17.4, 10.7²	2 yr
Shute 1984	Noturus flavipinnis	2.5-3.3	3.4²	150-278 (75-100, 88²)	7.8-15.5, 11.5² (0.60³)	2 yr
Jenkins and Musick 1979	Noturus flavipinnis	---	---	100-300	---	---
Bowman 1936	Noturus flavus	---	---	(197-200)	---	---
Clark 1978	Noturus flavus	---	---	200-500¹, 350²	---	---
Greeley 1929	Noturus flavus	---	3.5-4.0	(500¹)	---	---
Langlois 1954	Noturus flavus	---	---	767-1205 (767-1205, 973²)	---	---
Walsh and Burr 1985	Noturus flavus	1.9-3.4, 2.7²	2.6-4.0, 3.4² (3.0-3.6, 3.3²)	189-570, 278² (104-208, 174²)	20.2², 29.3³ (0.14², 0.6³)	3-4 yr

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Table 4.5 continued. Factors associated with gonadal maturation and reproduction of ictalurids.

Source	Species	Diameter of yolked ovarian oocytes-mm	Diameter of fertilized egg's chorion (yolk)-mm	Absolute fecundity (clutch size)	Female (male) GSI	Age or size at maturity
Thomerson 1966	Noturus funebris	2-3	---	114-192, 163²	---	---
Clark 1978	Noturus funebris	3.1-4.1, 3.4²	---	67-170, 111²	---	---
Jenkins and Musick 1979	Noturus gilberti	---	---	35-65	---	2 yr
Todd 1973	Noturus gyrinus	---	---	85	---	---
Menzel and Raney 1973	Noturus gyrinus	---	---	82-179, 122²	---	---
Bailey 1938	Noturus gyrinus	---	3.5	(117)	---	---
Evermann and Clark 1920	Noturus gyrinus	---	---	50-93, 72²	---	---
Clark 1978	Noturus gyrinus	3.1-3.9, 3.7²	---	28-107, 60²	---	---
Mayden and Walsh 1984	Noturus gyrinus	1.8-2.4, 2.0²	---	87-116, 99²	---	---
Wang and Kernehan 1979	Noturus gyrinus	---	2.8-3.2 (2.5-2.7¹)	(125-150)	---	---
Mahon 1977	Noturus gyrinus	---	---	43-160	---	1 yr
Whiteside and Burr 1986	Noturus gyrinus	1.1-2.4, 2.0²	(2.9-3.1, 3.0²)	48-323, 151²	3.4-22.6, 14.9² (1.11³)	1-2 yr
Mayden and Walsh 1984	Noturus hildebrandi	1.8-2.8, 2.3²	3.0-3.8, 3.4² (2.6-3.2, 2.8²)	17-38, 30² (11-27, 19²)	7.5-22.7, 13.9² (1.33³)	1 yr
Bowman 1932	Noturus insignis	2.1-2.3, 2.2²	---	53-223	---	---
Clark 1978	Noturus insignis	---	---	107	---	---
Fowler 1917	Noturus insignis	---	3¹	(200¹)	---	---
Clugston and Cooper 1960	Noturus insignis	4¹	---	---	---	2 yr

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Table 4.5 continued. Factors associated with gonadal maturation and reproduction of ictalurids.

Source	Species	Diameter of yolked ovarian oocytes-mm	Diameter of fertilized egg's chorion (yolk)-mm	Absolute fecundity (clutch size)	Female (male) GSI	Age or size at maturity
this study	Noturus insignis	3.4-4.4, 3.8[2]	5.5[2] (3.8[2])	42-207, 129[2] (44-198, 105[2])	4.5-14.6, 9.9[2] 00.34-0.70, 0.54[2]	---
Clark 1978	Noturus leptacanthus	2.9-4.3, 3.6[2]	5.5 (3.8)	14-45, 24[2] (13-25, 18[2])	---	---
Mayden and Walsh 1984	Noturus leptacanthus	1.9-2.6, 2.3[2]	---	28-59, 41[2]	---	---
Bowen 1980	Noturus miurus	---	---	(40)	---	---
Taylor 1969	Noturus miurus	---	---	(34-46, 38[2])	---	---
Hardman 1981	Noturus miurus	---	---	(35-95, 62[2])	---	---
Menzel and Raney 1973	Noturus miurus	---	---	31-143, 89[2]	---	---
Burr and Mayden 1982b	Noturus miurus	2.5-3.0, 3.0[2]	3.4-4.8, 3.5[2] (3.0-3.4, 3.2[2])	42-90, 66[2] (56-81, 66[2])	14.5[2] (0.89[3])	1-2yr
Trauth et al. 1981	Noturus munitus	---	---	50-70	---	---
Burr and Mayden 1982a	Noturus nocturnus	1.8-2.3, 2.0[2]	3.6-4.5, 3.9[2] (3.1-4.0, 3.3[2])	85-116, 102[2] (35-154)	5.2-8.9, 7.9[2] (0.62[2], 0.91[3])	1-2 yr
Mayden and Walsh 1984	Noturus phaeus	2.1-2.8, 2.5[2]	---	108-128, 118[2]	---	---
Moss 1981	Noturus placidus	---	---	32	---	---
Minckley and Deacon 1958	Pylodiis olivaris	2.6-3.4	---	6900-11300	---	4-6 yr 46-51 cm
Breder 1935	Pylodictis olivaris	---	---	(100000[1])	---	---

[1]Estimated value
[2]Mean value
[3]Maximum value

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madtoms into the laboratory during April year and held them at temperatures of ≈ 24 to 26 C and a photoperiod of ≈ 13.5 h. Some of those fish obtained spawning condition by the following February, but the percentage of fish in spawning condition was not reported. Brauhn and McCraren (1975) have shown that ovaries of the channel catfish can develop normally under a regime of constant, relatively low temperature (17 C) and short photoperiod (8 h light), but that degree of ovarian development may vary widely among fish. Peak GSI values occurred six months after the normal spawning season in their study. Davis et al. (1986) compared ovarian development among blinded, pinealectomized, blinded plus pinealectomized, and normal channel catfish. They found that fish in all groups developed mature ovaries, but that treatment groups matured later and at lower rates than control fish. They also found that fish exposed to constant warm temperature (21 C) and natural photoperiod matured one month earlier than fish exposed to natural temperature and photoperiod cycles. They concluded that reproductive cycling in the channel catfish is controlled by an annual internal oscillator that may be modified by environmental temperature.

Based on the results of Experiment UET-CWP, changes in temperature are not requisite to maturation of ovaries of margined madtoms collected after the spawning season, and maturation of captive females (based on GSI values and mean oocyte diameters) was not statistically different than that of wild fish. Gonadal development of males, however, was negatively impacted by the lack of a

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temperature cycle. The temperature and photoperiod cycles can effectively be compressed by at least by two months for the margined madtom, thereby providing 'ripe' fish before the onset of the normal breeding season, and effectively extending the breeding season for working with rare madtoms. Additional studies are necessary to help clarify the importance of temperature and photoperiod on maturation of the gonads of the margined madtom. Cycling of one or both does, however, seem to be necessary because madtoms held for several months under simulated spawning conditions after the conclusion of maturation experiments failed to maintain or to develop ripe gonads. In addition, dozens of other margined madtom females not used in maturation experiments, and held for over a year under constant warm temperature and long photoperiod only occasionally developed ripe gonads. Thus, regulation of photoperiod, and perhaps temperature, seems to help initiate and synchronize ovarian development in captive madtoms.

Many of the males in this study developed secondary sexual characteristics as spawning conditions approached. However, the GSI values of these fish were not different from those of fish sampled at other times, and motile sperm were never observed in any of the males. Injections of CPE increased the GSI of males, but the number of spermatozoa was lower in injected males. Vascularization of testes was greater in males injected with CPE, indicating that continuation of injections may result in development of the testes, and perhaps the production of motile sperm.

Seasonal Steroid and GSI Trends

The volume of plasma obtained from margined madtoms was sufficient to run duplicate RIA samples for 17β-estradiol or testosterone. However, little or no plasma remained after running 17β-estradiol samples because a volume of 200 μL was required, and females typically were smaller than males. Thus, steroid sampling of madtoms is restricted to one run of one hormone, and retesting or testing for additional hormones generally is not possible.

Changes in plasma steroid levels have been correlated with phases in the reproductive cycles of fishes. In females, growth of oocytes is principally dependent on an accumulation of yolk, the production of which is primarily a function of estrogen-dependent vitellogenin synthesis by the liver (Korsgaard and Petersen 1979). Estrogen levels typically peak just prior to the spawning season, and then decline during the prespawning period (Wingfield and Grimm 1977, Lamba et al. 1983, Zohar and Billard 1984). Lamba et al. (1983) speculated that lower 17β-estradiol and concurrent high testosterone levels may act synergistically to promote storage of gonadotropin in the pituitary for subsequent release as a surge at the time of oocyte final maturation and spawning.

Female margined madtoms in this study exhibited the expected rise in 17β-estradiol plasma levels in association with ovarian recrudescence. A peak in plasma 17β-estradiol occurred just prior to the spawning season; female GSI values for wild fish peaked approximately one month after 17β-estradiol (late April verses late May).

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The lowest GSI values (< 3) were associated with the lowest 17β-estradiol levels, intermediate GSI values (3 to 8) were associated with the highest levels of 17β-estradiol, and the highest (prespawning) GSI values (> 8) were associated with intermediate 17β-estradiol levels.

The range of plasma 17β-estradiol levels in this study (mean values of 1.5 to 7.3 ng/mL) were within the range of those reported for other ictalurids. For the channel catfish, mean values of approximately 2 to 12 ng/mL (Davis et al. 1986) and 2 to 40 ng/mL (MacKenzie et al. 1989) have been reported, and for the brown bullhead mean values of approximately 1 to 6 ng/mL (Burke and et al. 1984) and 1 to 8 ng/mL (Rosenblum et al. 1987) have been reported.

The seasonal pattern for 17β-estradiol in female margined madtoms is similar to that reported by Davis et al. (1986) for the channel catfish. They reported that GSI and plasma 17β-estradiol rose concurrent with GSI until GSI values peaked in early June at which time a decline in 17β-estradiol levels occurred. MacKenzie et al. (1989) reported that plasma levels of 17β-estradiol in female channel catfish were associated with an initiation of vitellogenesis, however, the peak value in their study occurred in February instead of May, and subsequent 17β-estradiol levels gradually declined until time of spawning. Thus, channel catfish in the study of MacKenzie et al. (1989) did not follow the generalized trend for teleosts previously described, or the pattern reported by Davis et al. (1986) for the channel catfish. Female brown bullhead also did not follow the generalized trend. Burke and et al. (1984) and

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Rosenblum et al. (1987) found two 17β-estradiol peaks for brown bullhead females; one just prior to the spawning season and the other toward the end of the spawning season. Peak GSI values occurred between the peaks. In addition, Rosenblum et al. (1987) found significant differences in seasonal 17β-estradiol patterns between consecutive years for the brown bullhead. Thus, there does not seem to be a uniform 17β-estradiol seasonal pattern among species of ictalurids, and it seems that annual variations within species also occur.

The pattern of gonadal development and range of GSI values of captive females were similar to those of wild fish, but the 17β-estradiol levels of captive fish were suppressed relative to the wild fish. Davis et al. (1986) also reported similar results for captive female channel catfish held under constant warm temperature (21 C) and natural photoperiod. They suggested that lack of a temperature cycle was the reason for depressed 17β-estradiol production. Because the margined madtoms in this study were exposed to a temperature cycle that mimicked that of nature, the reason for the difference in magnitude of 17β-estradiol production of captive verses wild fish is uncertain.

In male teleosts, a simple peak of testosterone has been measured during the prespawning phase of the annual cycle of most species (Wingfield and Grim 1977, Scott et al. 1980, Stuart-Kregor et al. 1981, Lamba et al. 1983). The seasonal testosterone levels parallel changes in the testicular cycle and GSI values; increases in testosterone levels coincide with active spermatogenesis.

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Male margined madtoms in this study exhibited a distinct seasonal pattern in plasma testosterone levels. Values gradually declined from November through the winter and then climbed rapidly in April, peaking in June. A rapid decline occurred at the conclusion of the spawning season. An increase in GSI values did not, however, parallel increases in plasma testosterone. There was no discernible correlation between GSI value and plasma testosterone levels. Maximum testosterone levels in male margined madtoms ($\approx$ 9 ng/mL) were approximately three times higher than those found in the brown bullhead (Burke et al. 1984, Rosenblum et al. 1987). Male brown bullhead exhibited dual peaks in plasma testosterone during the prespawning and early spawning season, and the seasonal peaks were correlated with high GSI values and increases in spermatogenic activity (Burke and Leatherland 1984, Burke et al. 1984, Rosenblum et al. 1987). Similar to females, male ictalurids do not seem to have a uniform seasonal pattern for reproductive steroids or gonadal development either within or among species.

The lack of a consistent relationship between GSI values and plasma steroid concentrations within and among species of ictalurids, indicates that the relationships are complex. More work with madtoms is necessary to determine whether multiple patterns among or within species of Noturus exist. At present, use of steroid levels as a tool to monitor gonadal condition of rare madtoms is not useful.

Recommendations for Future Work

Although control of the reproductive cycle of female madtoms is feasible, I cannot currently recommend attempts to control the reproductive cycle of rare madtoms in captivity, because methods to ensure production of viable sperm in males have not been developed. Thus, additional work with nonthreatened madtoms is requisite. I recommend working to maximize number of spawnings and subsequent reproductive output of females by exposing captive fish to compressed temperature and photoperiod cycles, a more in-depth examination of the spermatological process to ensure production of high quality sperm, and a closer examination of the relationships between steroids and the spawning cycle in madtoms, to better understand the reproductive biology of madtoms.

Gonads of female <u>Noturus</u> spp. do not readily mature when fish are exposed to a regime of constant photoperiod ($\approx$ 16 light per day) and temperature ($\approx$ 22 C). Based on the results of this study, changes in photoperiod, but not temperature, are necessary to promote gonadal maturation of a large proportion of captive <u>Noturus</u> spp. females. However, GSI values of males exposed to constant temperature and cyclic photoperiod were lower than those of males exposed to cyclic temperature and photoperiod. Consequently, I recommend using photoperiod and temperature cycles that mimic natural cycles to induce gonadal maturation of captive madtoms. Temperature and photoperiod cycles can be compressed by at least four months when fish are collected from the wild in late October or early November. All fish in

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this study were collected in the late fall. For future studies, I recommend collection of madtoms immediately after the spawning season. This would permit immediate exposure of captured fish to compressed temperature and photoperiod cycles, thereby maximizing the number of spawning cycles per annum. I recommend evaluation of three, five, and seven month cycles. I recommend disproportionately greater compression of the winter portion of the temperature and photoperiod cycles, because most gonadal development in ictalurids occurs when temperature is rising or is warm (Davis et al. 1986, Rosenblum et al. 1987, Mackenzie et al. 1989).

A diet of only moist pellet can be used to produce mature gonads of female madtoms, but the use of live food (i.e., meal worms or earth worms) to enhance condition and subsequent gonadal development should be investigated. Collection and survival of post-spawning madtoms should not prove problematic because they can be handled effectively at relatively warm temperature when sufficient precautions are taken. Precautions include not overcrowding fish in collection containers, prompt placement of captured fish into an aerated hauling tank, and addition of salt (0.2 %) and Furacin (10 mg/L) to the hauling water. Fish may also be anesthetized, but I have not found this to be necessary. Electrofishing is a satisfactory collection technique, because no madtom mortalities in this study were directly related to electrofishing.

The resorption of oocytes by females necessitates regular inspections of females to determine state of maturation. I do not currently recommend use of

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catheterization to determine diameters of oocytes and subsequent state of maturation, because catheterization may inhibit ovulation (see Chapter 5). I recommend examination of external characteristics of the female. The abdomen of prespawning fish will be distended, and large oocytes usually are visible through the wall of the abdomen. If females are judged to be in spawning condition they should be hormonally injected to determine whether they can be induced to spawn (see methods for inducing spawning in Chapter 5).

Methods to prolong the time to resorption of mature oocytes in females should also be examined. I suggest evaluating the effect of exposing prespawning females to low temperature (< 18 C) or prolonged darkness. Temperature below that requisite for spawning may postpone resorption of oocytes by inhibiting final maturation, and a short or absent photoperiod may extend the spawning season. Davis et al. (1986) reported blinded or pinealectomized channel catfish retained viable oocytes for one month longer than normal fish.

Production of viable sperm in captive male madtoms is difficult to control; consequently, I recommend an in-depth study on the biology of the male's reproductive cycle. The study should include histological examination of testes of wild fish throughout the year, and comparisons of the spermatological cycle of these fish with those of captive fish exposed to temperature-photoperiod regimes previously described. The efficacy of hormonal therapies to induce spermatogenic activity

should also be examined. Suggested types of hormonal therapies to be evaluated are discussed in Chapter 5.

Additional work should be done to clarify the relationships between steroid production and gonadal development in madtoms, because this would lead to a better understanding of the reproductive biology and requirements of madtoms. Based on the results of my study, I recommend use of standards that range from 0 to 30 ng/mL for 17β-estradiol, and 0 to 15 ng/mL for testosterone. Relatively large madtoms, such as the margined madtoms, should be used, because the size and subsequent plasma volume of madtoms is small. I recommend using less plasma per replicate, because this would permit additional replicates to be run, or testing for another steroid with the plasma remaining from each sample. Plasma volumes of 1/4 or 1/2 of those called for in the kits (50 μL for testosterone, and 100 μL for 17β-estradiol) are probably adequate, because the minimum sensitive range of the RIA kits used in this study was 0.008 ng/mL for 17β-estradiol and 0.04 ng/mL for testosterone. The steroid levels of fish collected from the wild should be compared with captive fish from maturation experiments. More frequent samples should be taken just prior to and during the spawning season, to better understand changes that occur during this critical period. Steroid levels should also be compared with gonadal state by determining GSI values, oocyte diameters, and spermatogenic stages of testes. The spermatogenic stages should be determined via histological examination.

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CHAPTER 5

Controlled spawning of <u>Noturus</u> <u>insignis</u>

Introduction

Several madtoms have been spawned in captivity (Wang and Kernehan 1979, Bowen 1980, Fitzpatrick 1981, Burr and Mayden 1982a, Mayden and Walsh 1984). A goal of this study was to develop a protocol for spawning rare madtoms in captivity through a critical evaluation of the literature and experiments conducted on a model species; the margined madtom, <u>Noturus</u> <u>insignis</u>. The second goal of this study was to develop a methodology that produces a high percentage hatch of madtom egg masses.

The organization of this chapter is by subsections that concentrate on tank-spawning experiments, induced-spawning experiments, and development of hatching techniques for egg masses of the margined madtom. Objectives are listed and briefly described in the subsequent subsections. Discussion of results is couched in terms of working with rare fishes, and generalizations to all species of madtoms are made where appropriate.

Tank-Spawning Experiments

Tank-spawning is desirable because it minimizes the need to handle fish, and subsequent handling-induced stress. Bowen (1980) spawned four of nine pairs of the brindled madtom, _Noturus miurus_, and Wang and Kernehan (1979) reported the spawning in aquaria by two pairs of the tadpole madtom, _Noturus gyrinus_. However, Clark (1978) was unable to spawn the speckled madtom, _Noturus leptacanthus_, in aquaria, and Fitzpatrick (1981) did not produce spawns of the slender madtom, _Noturus exilis_, or the brindled madtom in aquaria without use of hormonal injections. A second attempt by Bowen (1980) to spawn the brindled madtom in aquaria was unsuccessful, even though fish exhibited spawning behavior. The objective of this study was to determine whether captive margined madtoms could be tank-spawned by controlling temperature and photoperiod. Some experiments employed injections of low doses of hormones to promote gonadal maturation.

Induced-Spawning Experiments

Fitzpatrick (1981) induced spawning of one of five pairs of the slender madtom, seven of eleven pairs of the brindled madtom, and three of three pairs of the freckled madtom, _Noturus nocturnus_, using several injections of human chorionic gonadotropin (hCG) at 50 to 150 I.U. per fish. Mayden and Walsh (1984) spawned five of eight pairs of the least madtom, _Noturus hildebrandi_, and Burr and Mayden (1982a) spawned three pairs of the freckled madtom using several injections of

50 I.U. of hCG per fish. The channel catfish, <u>Ictalurus</u> <u>punctatus</u>, has been spawned successfully in aquaria when females were injected with 100 to 600 μg/kg of luteinizing hormone releasing hormone analogue (LHRHa), 660 to 1760 I.U./kg of hCG, or 3.0 mg to 4.4 mg/kg of carp pituitary extract (CPE, Busch and Steeby 1990). Other ictalurids have also been induced to spawn in captivity by injecting them with hormones (Sneed and Clemens 1960, Wallace 1967, Wallace 1972). The objectives of this study were to determine the effect of relatively high doses of injected hormones on final maturation of oocytes, and to ascertain whether hormonal injections can induce spawning of the margined madtom.

Key criteria used to assess spawning readiness of females were initial positions of germinal vesicles and diameters of oocytes, because they have proven valuable for other species (Conte et al. 1988, Rottmann and Shireman 1988). Changes in positions of germinal vesicles following hormonal injections were used to evaluate the efficacy of the treatments to induce final maturation of madtom oocytes. Number of fish that spawned was the primary criterion used to evaluate the effectiveness of hormonal treatments to induce captive spawning.

Hormonal Implants

Gonadotropins, steroids, and releasing hormones have been administered via cholesterol pellet and silastic tubing implants (Crim 1985). Implants of 17α-methyl-testosterone, LHRHa, or a combination of the two have been effective in inducing

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ovulation and spermiation (Lee et al. 1986a, Lee et al. 1986b), at dosages of ≈ 25 to 300 μg of LHRHa and ≈ 125 to 2500 μg of 17α-methyltestosterone per kilogram (Crim et al. 1983, Crim and Glebe 1984, Lee et al. 1986a, Lee and Tamaru 1988). The primary difference between hormonal implants and injections is that implants can result in a more extended, slow release of hormones. Thus, implants can be used to elevate levels of desired hormones for a period of weeks or months (Crim et al. 1988). The effects of hormonal injections typically last only for a few hours (Crim et al. 1988), and less stress is incurred by fish during pellet implantation compared to a prolonged series of injections (Weil and Crim 1983). The objective of this study was to determine the efficacy of hormonal implants to induce final maturation of the gonads and spawning of the margined madtom.

Hatching Egg Masses

Shute et al. (1990) reported that hatching newly spawned ova of the yellowfin madtom, Noturus flavipinnis, was difficult. Bowen (1980) reported that male brindled madtoms ate or rejected three of four egg masses spawned in aquaria. Clark (1978) found it difficult to hatch newly spawned egg masses of the speckled madtom collected from the wild. She also found that guardian males, collected over nests in the wild, ate the egg masses when they were used to incubate the eggs in captivity. Bowman (1936) found it difficult to hatch ova of the margined madtom. However, Mayden (1980) reported hatching success rates of 39 to 75 % for slender madtom egg

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masses; Burr and Mayden (1982a) reported 100 % hatch of an egg mass of the freckled madtom; and Burr and Mayden (1982b) reported an 86 % hatch of four clutches of brindled madtom eggs. The objective of this study was to test various methods for hatching egg masses of madtoms using the margined madtom as a model species.

Materials and Methods

Fish used in all experiments were captured by electrofishing in two tributaries of the New River, Virginia; Wolf Creek in Bland County and Toms Creek in Montgomery County. Preliminary trials indicated that adult margined madtoms will begin to feed on moist pellets 4 or 5 d after capture. They did not readily accept crushed trout chow. Consequently, fish used in all experiments were fed a 3 mm diameter moist pellet once or twice daily. The moist pellet was prepared by grinding whole, fresh or frozen fish with commercial trout or catfish feed in a commercial meat grinder equipped with a 3 mm extruder plate. Shortly after capture, fish were prophylactically treated for disease for 3 to 4 h with salt (0.2 %) and Furacin (5 to 10 mg/L), and formalin for 3 to 4 h (25 mg/L) or for 1 h (250 mg/l). They were treated periodically thereafter.

Tank-Spawning Experiments

Three types of tanks were used in tank-spawning experiments. A circular, metal tank (1.8 m diameter X 0.6 m height) was used outdoors. Consequently, fish in this tank were exposed to natural temperature and photoperiod regimes. The tank bottom was covered with pea size gravel, and a submersible pump created a circular current within the tank. Shelters consisted of large concrete blocks and half-sections of 102 mm I.D. PVC pipe that were 250 to 300 mm long.

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Four indoor experimental units consisting of five 110 L aquaria each, were used in five experiments. Each unit drained into a 175 L under-gravel biofilter, and received a steady, small input of municipal water that had been passed through a bed of activated charcoal for dechlorination. Water from the biofilter was pumped to a head tank from which it was recirculated to aquaria. Each aquarium was supplied with two air stones and a half-section of 102 mm I.D. PVC pipe that was 250 to 300 mm long. The PVC shelters were placed atop small piles of pea gravel. Air flow was adjusted to provide a moderately strong circulation of water within aquaria. Water temperature in each unit was controlled by a thermostat and 220 volt heating element. Temperature was held constant in one trial at 25, at 26 C in another, and in the others it was gradually elevated from 20 to 26 C, 23 to 28 C, or 24 to 28 C. Translucent ceiling panels provided natural photoperiod.

A third tank type was a 1 m X 10 m indoor concrete raceway that was converted to an artificial stream for one experiment. Concrete blocks and gravel from a local stream were placed in the bottom of the raceway to create three riffle-pool series. Each riffle-pool series was isolated from the others by a screened divider, and a current was created by pumping water from one end to the other with two one-horsepower centrifugal pumps. The maximum depth was $\approx$ 20 cm.

Fish in four of the five aquaria experiments and the artificial stream experiment were injected with low doses of a hormone (10 μg/kg of LHRHa; des-Gly[10]-[DAla[6]], ethylamide) and a dopamine blocker (1 mg/kg of pimozide) to

encourage development of gametes and spawning behavior. Fish in the outdoor experiment were not injected. The circular tank experiment lasted 60 d, the artificial stream experiment ran 58 d, and the aquaria experiments were from 10 to 30 d in length.

Induced-Spawning Experiments

Three tank systems were used in induced-spawning experiments. One consisted of three 110 L aquaria that drained into a large Living Stream (500 L). The Living Stream received a steady, small input ($\approx$ 1 L/min) of activated charcoal-filtered municipal water, and water from the tank was recirculated to aquaria by a submersible pump. Water temperature was controlled with a Frigid Units water chiller and a Blue-M submersible heater. No shelters were provided in these tanks.

A second system consisted of twelve 110 L aquaria that drained into a large Living Stream. Water from the Living Stream was recirculated to aquaria by a submersible pump. Water temperature was controlled by inputs of heated or unheated well water that were directed by a computer-linked thermostat. Each aquarium was equipped with an under-gravel filter (depth of gravel was $\approx$ 75 mm), and a slab-rock approximately the same width as the tanks served as a shelter (this is the same system used in Experiment CNT-CNP of Chapter 4).

Living Streams were the tanks used in the third system. Two of these were used, and each received a small, steady input ($\approx$ 1 L/min) of activated charcoal-

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filtered municipal water. Four large slab-rocks (≈ 30 to 40 cm diameter), each placed over 12 L of pea gravel, served as shelters for the madtoms in each tank.

Photoperiod was 16 h light in all studies. Photoperiod was controlled by a set of incandescent lights and timers, or a computer-regulated rheostat. Light intensity at the beginning and end of each day was gradually changed over a period of 0.5 to 1 h to preclude startling of fish by sudden changes in light.

The hormones used in all induced-spawning experiments were CPE, hCG, and LHRHa. A dopamine blocker, domperidone (dom), was injected concurrently with LHRHa (LHRHa + dom). The hormones CPE and hCG also were injected concurrently (hCG + CPE). A few fish were given only CPE injections. If combined treatments were used (LHRHa + dom or hCG + CPE), the chemicals were injected separately in consecutive injections.

Males and females were injected with similar hormonal dosages. In all cases, the first injection (primer injection) was of a lower dose than subsequent injections. The first high dose injection was always given on the same day as the primer injection, and subsequent high dose injections were given on consecutive days thereafter.

All hormones were dissolved in 0.2 mL of 0.6 % NaCl solution and injected with a 26 gauge hypodermic needle and tuberculin syringe. Injections were given intraperitoneally at the base of the pelvic fin, because back-flow of injected solutions sometimes occurred when injections were given intramuscularly.

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Hormonal dosages that can induce spawning of margined madtom are not known. Consequently, I made no attempt to regulate dosages of hormones on a per kilogram basis for each fish. Instead, dosages were computed on a per fish basis, and the universal injection dosages were based on a 25.0 g fish. Dosages for primer injections (based on a 25.0 g fish) were 20 μg/kg of LHRHa, 2 mg/kg of domperidone, 200 I.U./kg of hCG, and 5 mg/kg of CPE. High dosages were 200 μg/kg of LHRHa, 20 mg/kg domperidone, 2000 I.U./kg of hCG, and 20 mg/kg of CPE. Total lengths of females were measured and recorded, and a length-weight equation, developed from margined madtoms used in maturation experiments (see Chapter 4), was used to estimate weights (weight (g) = -10.77 + 2.86 * natural log of total length (mm)). Subsequent dosages per kilogram for each fish were then calculated using these weights. The total lengths were also used as marks to identify individuals. If lengths among fish used in a spawning trial were similar, fins were clipped to permit identification of individuals.

Weights of females that spawned were compared with those that did not spawn for each treatment (hCG +CPE or LHRHa + dom) with a t-test. This test indirectly tested for differences in the efficacy of different hormonal dosages to induce spawning of the margined madtom, because dosage was directly proportional to weights of females. The range, mean, and standard deviation of hormonal dosages that produced spawns were determined for each treatment.

Table 5.1. Summary of induced-spawning experiments.

Experiment number	Tank type[1] (cover)	Pairs per tank (N[2])	Oocytes sampled	Temperature (C)
1	110 L aquaria (none)	2-4 (50)	Yes	22-24
2	110 L aquaria (slab-rocks)	1 (67)	No	23
3	500 L rectangular (slab-rocks)	3-4 (14)	No	23
4	110 L aquaria (none)	2 (8)	No	23

[1]See text for a more detailed description of tanks and other physical conditions
[2]Total number of pairs injected

Four experiments were carried out in this study (Table 5.1). Fish in all experiments were anesthetized with MS-222 (25 to 50 mg/L) prior to sampling of oocytes or injection of hormones. In Experiment 1, 44 pairs of margined madtoms captured from the wild during the spawning season (early June to mid-August) were given hormonal injections. Six females that had developed mature ova in captivity were also injected. Twenty-one pairs of fish were injected with hCG + CPE, and 24 pairs were injected with LHRHa + dom. Five pairs were injected with CPE only. The maximum number of injections given to fish in this experiment was four. The three-tank system was used in this study, and two to four pairs of fish per tank were hormonally injected in each trial. In this study, oocytes were sampled to determine the effects of hormonal injections on maturation of oocytes.

Samples of oocytes were taken with a catheter from every female in Experiment 1. The catheter was a short section of 4.2 mm I.D. and 5.6 mm O.D. clear, flexible vinyl tubing attached to a syringe. Oocytes were extracted by inserting the tubing into an ovary via the ovipore and then applying suction. Positions of germinal vesicles and diameters were determined for three to nine oocytes (usually five or six). Oocyte samples were taken at the time of every injection, except the second, and on the day following the final injection.

Germinal vesicles were viewed under a dissecting microscope at 10X after oocytes had been cleared in a solution of ethanol (60 %), formalin (30 %), and glacial acetic acid (10 %). This solution was used because it is superior to several others for clearing oocytes of the margined madtom (Stoeckel and Neves 1992). An ocular micrometer was used to measure the shortest distance from the germinal vesicle to the periphery of the oocyte. Oocytes were repositioned if necessary to obtain the measurement. The diameters of oocytes were measured at the same time that germinal vesicle positions were determined. The 'germinal vesicle ratio' was calculated by dividing the shortest distance from the germinal vesicle to the periphery of an oocyte by the diameter of the oocyte. Oocytes were considered mature if the germinal vesicles were not visible in greater than one-half of the sampled oocytes.

Oocytes were not sampled from fish in Experiments 2, 3, or 4. In Experiment 2, one pair of hormonally-injected fish was used per tank; in Experiment 3, three or four pairs were used per tank; and in Experiment 4, two pairs of fish were used per

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tank. The number of pairs of fish injected with LHRHa + dom was 34, 7, and 4 for Experiments 2, 3, and 4, respectively. The number of pairs of fish injected with hCG + CPE was 25, 7, and 4 for Experiments 2, 3, and 4, respectively. Eight pairs of fish were injected as controls in Experiment 2. Fish in these experiments were given as many as five injections. The twelve-tank system was used in Experiment 2, Living Streams were used in Experiment 3, and the three-tank system was used in Experiment 4.

Injected females in all experiments had well developed ovaries, and all injected males had well developed head musculature. Well developed head musculature is characteristic of male madtoms that are preparing or guarding nests (i.e., breeding males, Burr and Mayden 1982a, Burr and Mayden 1982b, Mayden and Walsh 1984). Females were examined to see if they had ovulated at the time of each injection or at the end of each trial by gently squeezing their abdomen.

Based on the outcome of induced-spawning trials, females were placed into one of five categories. Category one included fish that died, and the other four described the fate of the oocytes. They were: category two, oocytes unovulated and atretic; category three, oocytes unovulated but not atretic; category four, oocytes mature and tank-spawned; and category five, oocytes mature and strip-spawned.

Changes in mean germinal vesicle ratios among injection times were used to assess the efficacy of hormonal treatments to induce final maturation of oocytes in Experiment 1. A general linear model (GLM) was used to test for statistical

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differences among the treatments. Initial mean germinal vesicle ratios and initial diameters of oocytes were compared with a GLM or a t-test to determine if they differed among fish given different hormonal treatments, or between those of fish in which maturation of oocytes occurred and those in which maturation did not occur. Mean times between successive injections of fish that spawned and those that did not in Experiments 2, 3, and 4 were statistically compared with a GLM. It was blocked by injection number, because they were known to vary with time. If GLM results were significant, multiple comparisons were made to determine where statistically significant differences existed.

Hormonal Implants

Silastic tubing and cholesterol-based implants were used in this study. Males received silastic tubing implants that contained 17α-methyltestosterone similar to those described by Lee et al. (1986c). One centimeter lengths of silastic tubing (1.45 mm I.D., 1.925 mm O.D.) were sealed at one end with medical grade elastomer and allowed to cure. A solution comprised of 10 mg of 17α-methyltestosterone per 0.1 mL of 100 % ethyl alcohol was mixed with 0.9 mL of castor oil. Tubes in which the sealed end had cured were filled with 12.5 μl of the hormonal solution, and then the open end was also sealed with elastomer. The dosage per implant was ≈ 125 μg.

Females received cholesterol implants that contained luteinizing hormone releasing hormone analogue (LHRHa), similar to those described by Crim et al.

(1988). A solution of 0.2 mg of LHRHa dissolved in 0.3 mL 50 % ethyl alcohol was mixed with 190 mg of cholesterol and 10 mg of cellulose. The mixture was warmed for approximately 1 h at 30 C and then pressed into 3 mm diameter X 2.5 mm long pellets with a Parr pellet press. The dosage per pellet was approximately 25 μg. Implants without hormones (sham implants) were also prepared for both types of hormones. All implants were stored frozen until use.

Male implants were inserted in the abdominal cavity by puncturing the abdominal wall with a 14 gauge needle, inserting the implant, and then sealing the opening with super glue (Nemetz and MacMillan 1988). Female implants were inserted into the oviduct and then pushed into an ovary with a short section of small diameter, clear, flexible vinyl tubing. Ovarian implants have proven effective in inducing ovulation of female rainbow trout, Oncorhynchus mykiss (Crim et al. 1983).

In the first of two experiments, 20 fish obtained at the completion of Experiment CNT-CNP were used. These fish had been exposed to compressed temperature and photoperiod cycles (see Figure 4.4, Chapter 4). Temperature and photoperiod during the implant experiment were held constant at 24 C and 16 h of light, respectively. Five females and five males received hormonal implants, and five females and five males received sham implants. Ovarian samples were taken from all females with a catheter at the time of the implants, and the diameters and germinal vesicle positions of oocytes were determined. Single pairs of fish were placed into the 110 L aquaria system used in Experiment CNT-CNP of Chapter 4.

After 35 d the GSI of all fish was determined, and the diameters of samples of oocytes were measured.

In the second experiment, 12 fish that had been held in captivity for over a year at constant photoperiod (16 h light) and temperature ($\approx$ 22 C) were used. Three males and three females received hormonal implants, and three males and three females received sham implants. They were implanted using the methods of the first experiment. Hormonally-implanted fish and sham-implanted fish were placed into separate sections of the oval raceway described in Chapter 4. Three slab-rocks, each placed over 12 L of gravel, served as shelters in each section. Fish were exposed to constant photoperiod (16 h light) and temperature (22 C) for 30 d. The GSI values of all fish were then determined, and diameters of oocytes were measured.

Mean GSI values were compared between treatment and control groups at the end of each experiment with a t-test. Initial and final mean diameters of oocytes were also compared between treatment and control groups in each experiment with a t-test.

Hatching Egg Masses

Ovulated oocytes of females that had not tank-spawned, were stripped into plastic pans filled with water from the spawning tank. Sperm, obtained from macerated testes dissected from males, was then mixed with the water and ova. An

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air stone was placed in the pan to provide aeration and circulate water around the ova. Water was changed, and dead eggs were removed and counted twice daily.

A variety of methods to hatch egg masses collected from local streams or tank-spawns were evaluated (Table 5.2). The temperature ranged between 22 and 25 C during most egg hatching trials. The water temperature was elevated to 28 to 30 C for Methods 9 and 10. Dead eggs were removed with a pair of forceps at least twice daily for all methods. Malachite green and formalin were used to treat several egg masses. A dip treatment of malachite green (60 mg/L for 60 sec) was used on some egg masses in Methods 1, 6, and 7. For the dip treatment, egg masses were transferred (under water) to glass crystallizing dishes (90 mm diameter X 50 mm deep), most of the water was decanted, the malachite green solution was added, decanted after 60 sec, and the egg masses were then returned to the tank. Some egg masses incubated by Method 7 were treated with 5 mg/L malachite and 10 mg/L of formalin directly in the tank. Because water flow into tanks was $\approx$ 1 L per minute, the chemicals were gradually diluted.

The time to hatch and temperature during the incubation period of one tank-spawned egg mass and several freshly laid egg masses collected from the wild were used to estimate the number of degree-days required to hatch embryos of the margined madtom. Based on observations of the developmental process of tank-spawned egg masses, the egg masses collected from the wild were from 0 to 1 d old. Consequently, 0.5 d was added to the total incubation time of egg masses collected

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Table 5.2. Summary of methods used to hatch egg masses of <u>Noturus insignis</u>.

Method number	Incubation chamber	Basket type	Na$_2$SO$_3$ treated	Water exchange	Aeration
1	38 L aquaria	Crystallizing dish[1]	No	≈ 1 L/min	Air stones atop dish
2	50 L plastic pan	PVC ring[2]	No	2X daily	Air stones below ring
3	50 L plastic pan	PVC ring	Yes[3]	2X daily	Air stones below ring
4	4 L plastic pan	None	Yes[3]	2X daily	Air stones in pan
5	4 L plastic pan	None	No	2X daily	Air stones in pan
6	7000 L Oval raceway	12 L Plastic pan	No	≈ 2.5 L/min	Constant water flow
7	19 L Bucket or 38 L ice chest	None	No	2X daily	Air stones in ice chest or bucket
8	38 or 110 L aquaria	4.5 mm square mesh plastic screening	No	≈ 1 L/min	Air stones below basket
9	110 L aquaria	6.0 mm square mesh plastic screening	No	≈ 1 L/min	Air stones below basket
10	110 L aquaria with undergravel filter	Atop pea gravel	No	≈ 1 L/min	Constant water flow
11	7 L hatching jar	None	Yes[4]	≈ 4 L/min[5]	Constant water flow

[1]Circular glass dishes (90 mm diameter X 50 mm deep)
[2]50 mm high rings of 75 mm I.D. diameter PVC with plastic window screening glued to the bottom to support egg masses
[3]Held in a 1.5 % solution (Dorman 1986) long enough to dissociate egg mass (> 20 min)
[4]Held in a 1.5 % solution long enough to remove organic matrix, but not dissociate egg mass (10 to 15 min)
[5]Flow rate at which egg masses were lightly tumbled

from the wild.

Results

Tank-Spawning Experiments

Low level injections of hormones did not seem to facilitate the spawning process, and no spawnings of margined madtoms were observed in any of the tank-spawning experiments (Table 5.3). All pairs of margined madtoms created 'nests' by picking up gravel with their mouths and depositing it at the openings of the PVC shelters. Fish left shelters to feed and when it was dark, at which time they would swim about the tank. Fish fed well in all trials and did not, in general, seem to be stressed. However, in two of the aquaria experiments disease outbreaks occurred (due to Icthyopthirius multifiliis), and fish obviously were stressed toward the end of the experiments. In addition, it was difficult to find and capture fish in the artificial stream, because fish wedged themselves in small crevices. This sometimes resulted in prolonged attempts to capture fish for injections.

Occasionally males were observed chasing females; this sometimes resulted in a circling pattern of the pair of fish with the female usually, but not always, in the lead. Occasionally this behavior persisted for more than ten minutes, and it was punctuated by frequent pauses. During the pause a male or female would rub or nudge the other fish. Some fish (males and females) circled independent of the other fish. The caudal embrace, a behavior that precedes egg deposition (Fitzpatrick 1981), was never observed.

Table 5.3. Results of tank-spawning experiments with <u>Noturus</u> <u>insignis</u>.

Tank type (number of spawns)	Temperature (C)	Trial length (d)	Day of hormonal[1] injection	Number dead
Circular (0)	15-30	60	None	4 of 9
Raceway (0)	20-24	58	9,12	1 of 18
Aquaria (0)	20-26	15	7,9,12	0 of 20
Aquaria (0)	23-28	30	22	8 of 15
Aquaria (0)	25	12	None	1 of 21
Aquaria (0)	26	11	1	3 of 9
Aquaria (0)	24-28	10	1,5,7	6 of 28

[1]LHRHa (10 µg/kg) + pimozide (1 mg/kg)

Most experiments were terminated when it became obvious that females were resorbing their oocytes or when it was apparent that disease was impacting the health of fish. Health of fish at the beginning of all experiments was excellent, and all fish fed well. Thus, most mortality of fish occurred near the end of experiments because of disease outbreaks.

Table 5.4. Comparison of efficacy of hormonal treatments to induce oocyte maturation (germinal vesicle breakdown) and spawning of <u>Noturus</u> <u>insignis</u>. Values are percent of fish in each category.

Hormonal treatment	Number of fish	Injection number at which oocytes matured				Percent that spawned
		1[1]	3	4	None	
hCG+CPE	20	10	65	15	10	0
LHRHa+dom	23	17	39	17	26	0
CPE	5	10	80	0	20	0
All	48	12	54	15	19	0

[1]Time at injection 1 equals 0 h

Induced-Spawning Experiments

Temperature ranged from 22 to 24 C in Experiment 1, and was held constant at 23 C in Experiments 2, 3, and 4. The caudal embrace was observed numerous times in Experiment 1. The male always initiated the embrace, and frequently, but not always, the female responded by embracing the male. Males sometimes embraced different females in succession, and sometimes embraced other males. However, no fish tank-spawned or were strip-spawned in Experiment 1, even though oocytes matured in 81 % of females following hormonal injections (Table 5.4). Oocytes in 66 % of all injected fish matured by the time of the third injection.

Initial germinal vesicle ratios were similar for fish of all hormonal treatment groups (Table 5.5, p = 0.88). The statistical probability that the rate of germinal vesicle migration rates differed among hormonal treatments (hCG + CPE and

Table 5.5. Comparison of germinal vesicle (GV) ratios, and diameters of oocytes at different injection times for each hormonal treatment. Values are the mean ± one standard deviation (N).

Parameter measured and Injection number[1]		Hormonal treatment		
		CPE	hCG+CPE	LHRHa+dom
GV ratios	1	0.11 ± 0.05 (5)	0.12 ± 0.05 (21)	0.12 ± 0.08 (24)
	3	0.02 ± 0.03 (5)	0.02 ± 0.04 (21)	0.05 ± 0.06 (23)
	4		0.02 ± 0.03 (17)	0.04 ± 0.07 (16)
Diameter of oocytes (mm)	1	3.89 ± 0.10 (5)	3.65 ± 0.31 (21)	3.70 ± 0.37 (24)
	3	3.89 ± 0.22 (5)	3.92 ± 0.41 (21)	3.88 ± 0.35 (23)
	4		3.94 ± 0.32 (17)	3.93 ± 0.43 (16)

[1]No oocytes were sampled at the time of injection 2

LHRHa + dom, blocked for time period) was 0.19, and this implies that all hormonal treatments are equally effective in inducing final maturation of margined madtom oocytes. Initial positions of germinal vesicles influenced the time at which oocytes matured (Table 5.6). In fish with germinal vesicles closer to the periphery of oocytes, the oocytes tended to mature earlier than those in which germinal vesicles were further from the periphery. Oocytes did not mature in any fish when the initial germinal vesicle ratio was > 0.20, and the smallest initial germinal vesicle ratio of

Table 5.6. Metrics associated with the maturation of oocytes of <u>Noturus insignis</u> following hormonal injections.

Number of fish	Initial GV ratio mean[1] ± SD[2] (range)	Initial diameter (mm) mean ± SD (range)	Diameter (mm) at time of maturation mean ± SD (range)	Injection number at time of maturation[3]
6	0.02[a] ± 0.02 (0.00-0.04)	3.9[a] ± 0.4 (3.5-4.4)	3.9 ± 0.4 (3.5-4.4)	1[4]
26	0.11[a] ± 0.03 (0.07-0.19)	3.8[ab] ± 0.2 (3.5-4.2)	4.0 ± 0.2 (3.5-4.5)	3
7	0.17[b] ± 0.04 (0.11-0.20)	3.6[bc] ± 0.2 (3.4-4.0)	4.0 ± 0.3 (3.7-4.5)	4
9	0.18[c] ± 0.07 (0.10-0.33)	3.4[c] ± 0.5 (2.6-4.0)	3.6 ± 0.5 (3.0-4.1)	None[5]

[1]Means with the same letter are not statistically different ($\alpha = 0.05$)
[2]One standard deviation
[3]Samples of oocytes were considered mature if germinal vesicles were not visible in greater than one-half of the oocytes
[4]Time at injection 1 equals 0 h
[5]Ratios and diameters at time of last sample (after injection 4), oocytes did not mature

oocytes of a fish in which oocytes did not mature was 0.10.

The initial mean diameters of oocytes did not vary statistically among hormonal treatment groups ($p = 0.38$, Table 5.5), but they did vary between fish in which oocytes matured and those in which they did not ($p < 0.001$, Table 5.6). The initial mean diameter of oocytes of females in which oocytes matured was 3.8 ± 0.2 mm, and that of females in which oocytes did not mature was 3.4 ± 0.5 mm. Oocytes that were larger at the beginning of trials tended to mature earlier than those that were smaller. The range of mean oocyte diameters at the time of the first injection for females in which oocytes matured was 3.4 to 4.4 mm. Oocytes of four

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of the nine females without oocyte maturation were smaller than 3.4 mm (range = 2.6 to 3.3 mm). Diameters of oocytes increased an average of 0.23 mm for all fish with maturing oocytes, from the first injection to the fourth injection. The greatest increase in mean diameter of oocytes was for fish that matured at the time of injection 4 (0.4 mm). The mean diameter of oocytes at the time of maturation was 4.0 ± 0.3 mm (N = 39, range = 3.5 to 4.5 mm).

In Experiment 2, 44 of 59 (75 %) hormonally-injected females spawned or were assumed to have spawned; none of the control fish spawned (Table 5.7). Six fish were strip-spawned; tank-spawning was verified for 19 fish; and another 19 were assumed to have tank-spawned. Because all females were gravid at the time of the first injection, those that were no longer gravid at the time of the next scheduled injection were assumed to have tank-spawned even if no ova were observed in the tank. An examination of guts of fish in several of these tanks indicated that eggs were been consumed by the male or female.

None of the strip-spawned ova developed embryos. Eleven of the 19 tank-spawned egg masses were removed from nests and incubated. Embryos did not develop in six of these egg masses. Embryos were identifiable in ova of the other five egg masses within two days of the time of spawning. Seven of the eight egg masses that were not removed were eaten by the male or female (the female had been removed in four of the tanks after spawning had occurred). The male guarding the eighth egg mass died three days after spawning, and the egg mass had turned white.

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Table 5.7. Results of experiments to induce spawning of <u>Noturus</u> <u>insignis</u>. Values are number of fish in each category.

Experiment number	Hormones injected	Results of trials				
		Female died	Oocytes unovulated	Oocytes atretic	Strip-spawn	Tank-spawn
1	hCG+CPE	1	9	11	0	0
	LHRHa+dom	1	12	11	0	0
	CPE	0	0	5	0	0
2	hCG+CPE	1	2	2	3	6+11[1]
	LHRHa+dom	1	6	3	3	13+8[1]
	Water	0	8	0	0	0
3	hCG+CPE	1	1	1	3	1
	LHRHa+dom	0	0	0	6	1[1]
4	hCG+CPE	1	0	2	1	0
	LHRHa+dom	0	1	0	3	0

[1]Includes fish that were known to have tank-spawned + those that were assumed to have tank-spawned

Embryos were not observed in this egg mass or one other that was examined two days after spawning. The latter egg mass was unusual because many empty chorions were visible within the egg mass.

In Experiment 3, one fish tank-spawned, two were assumed to have tank-spawned, and eight were strip-spawned (Table 5.7). Thus, 86 % of the females in this experiment ovulated following hormonal injections. One female died on day five in one trial. The only tank-spawned egg mass observed in this experiment was lying

exposed near a corner of the Living Stream, and guarded by a male. This egg mass was eaten by the next day. None of the strip-spawned ova developed into embryos.

In Experiment 4, four of the eight hormonally-injected females ovulated and were strip-spawned (Table 5.7). One female died on day three. None of the strip-spawned ova developed into embryos.

The total number of females injected in all experiments was 139. The number of hours from time of capture to first injection averaged 24 ± 22 h (range = 0 to 73 h) for fish injected in Experiment 1, and 93 ± 108 h (range = 3 to 360 h) for fish injected in Experiments 2, 3, and 4. Because no fish spawned in Experiment 1 ($N = 50$), they are excluded from further analyses. Fish that died in Experiments 2, 3, and 4 ($N = 4$), and control fish injected with water ($N = 8$) are also excluded from the analyses. Fifty-nine of the 77 remaining females either tank-spawned or were strip-spawned (77 %). They ranged in length from 100 to 182 mm, with a mean length of 138 ± 20 mm. The equation to convert total length to weight is, ln weight (g) = -10.77 + 2.86 * ln length (mm) ($N = 242$, $R^2 = 0.87$). The estimated weight of the average female used in induced-spawning experiments was 30.0 ± 9.8 g, and the estimated range in weights was 11.3 to 50.0 g. Thus, the dosages of hormones given to an average female were slightly lower than those listed in the Materials and Methods section for a 25 g fish, and estimated dosages ranged from 45 % to 200 % of the values listed in the Materials and Methods section for a 25 g fish.
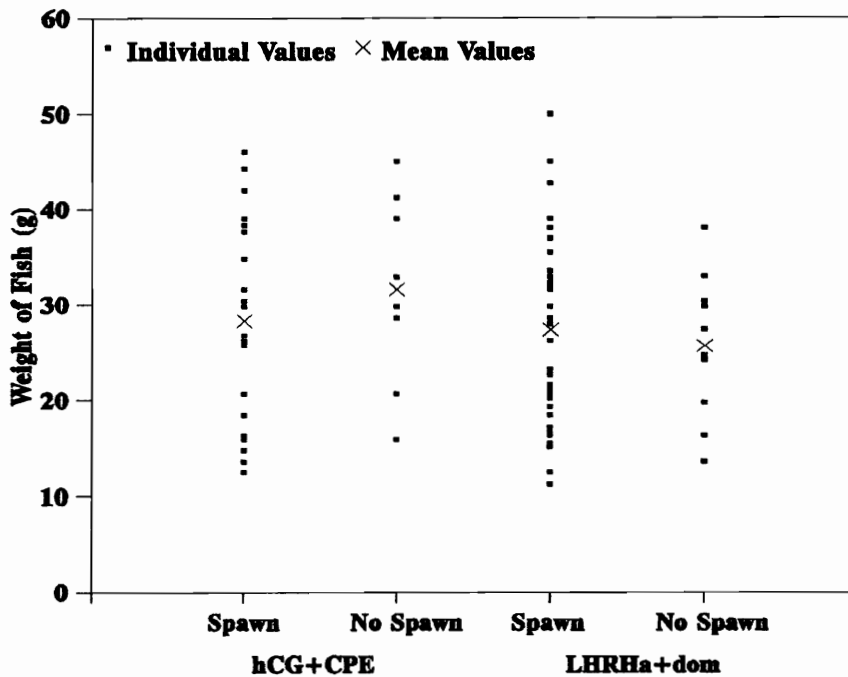
Figure 5.1. Weights of <u>Noturus</u> <u>insignis</u> females that spawned following hormonal injections.

The mean weight of females that spawned following injections of hCG + CPE was 28.4 ± 10.4 g (N = 25), and for those that did not spawn it was 31.6 ± 9.4 g (N = 8, Figure 5.1). The mean estimated weight of females that spawned following injections with LHRHa + dom was 27.5 ± 9.9 g (N = 34), and for those that did not spawn it was 25.7 ± 7.2 g (N = 10, Figure 5.1). Statistical comparisons within treatments indicated that the weights of females that did or did not spawn were not significantly different for either the hCG + CPE treatment (p=0.45) or the LHRHa + dom treatment (p=0.62). The effectiveness of the two treatments to induce spawning of the margined madtom was considered equal because 34 of 44 females

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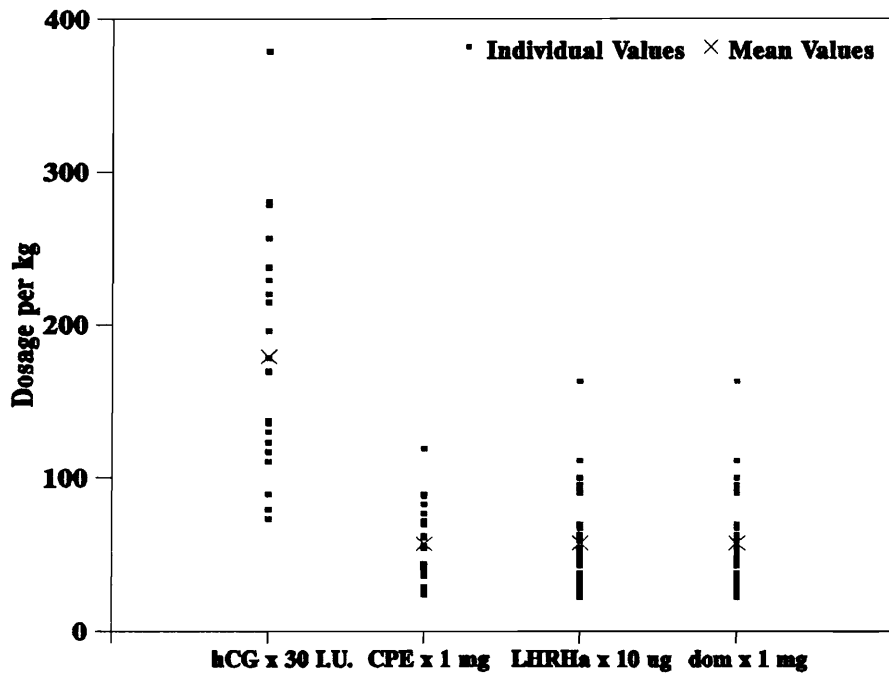
Figure 5.2. Dosages of hormones that induced spawning of female <u>Noturus</u> <u>insignis</u>.

injected with LHRHa + dom spawned (77 %), and 25 of 33 females injected with

hCG + CPE spawned (76 %). Thus, within the range of values tested in this study,

all dosages of hormones were equally effective in inducing spawning of captive

margined madtoms.

The dosages of hormones that induced spawning in the margined madtom

ranged from 2375 to 10035 I.U./kg of hCG and 25 to 156 mg/kg of CPE for the hCG

+ CPE treatment, and from 233 to 1256 μg/kg of LHRHa and 23 to 126 mg/kg of

dom for the LHRHa + dom treatment (Figure 5.2). The mean effective dosages

were 5256 ± 2037 I.U./kg of hCG and 58 ± 28 of CPE for the hCG + CPE

treatment, and 554 ± 210 μg/kg of LHRHa and 55 ± 21 mg/kg of dom for the

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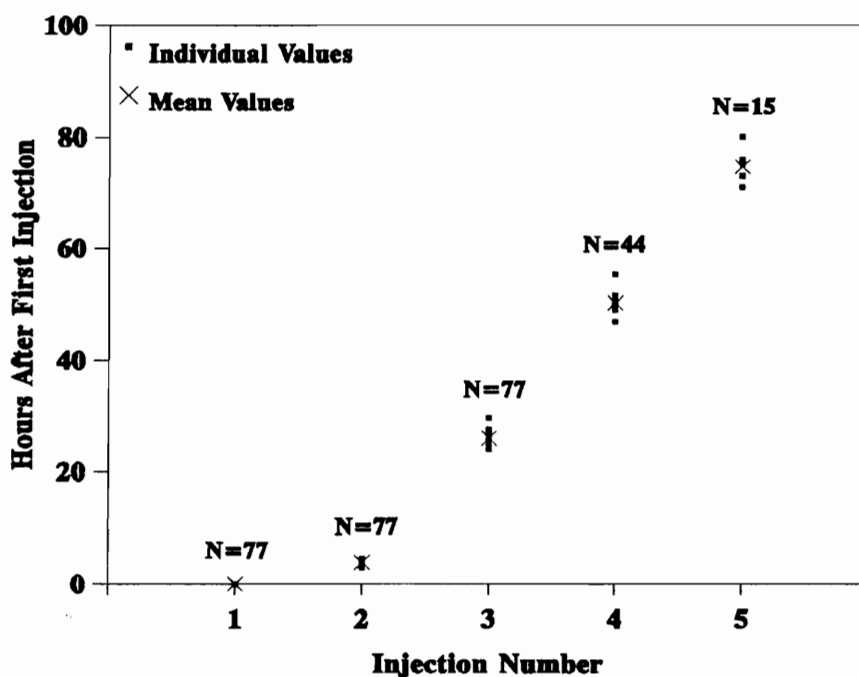
Figure 5.3. Time of hormonal injections for all spawning trials.

LHRHa + dom treatment.

All 77 females were given at least three injections; 44 were injected four times, and 15 were injected five times. The range of times within which fish were given successive injections was relatively narrow (Figure 5.3 and Table 5.8). The statistical probability that injection times did not vary among spawners and nonspawners was 0.95. The mean time of successive injections relative to that of the first injection (time = 0 h) was 3.9 h for injection 2 (N = 77), 26.0 h for injection 3 (N = 77), 50.3 h for injection 4 (N = 44), and 74.6 h for injection 5 (N = 15, Table 5.8).

The time to ovulation was similar for fish that were injected with LHRHa + dom or hCG + CPE. Most fish (49 %) were strip-spawned or tank-spawned at the

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Table 5.8. Comparison of time between injections of fish that spawned and those that did not, and of time to ovulation for fish that received hormonal injections of LHRHa + dom or hCG + CPE.

Injection number	Hours after first injection		Number of tank spawns (strip spawns)	
	Spawners (N)	Nonspawners (N)	LHRHa + dom	hCG + CPE
2	3.9 ± 0.3^{1} (59)	3.9 ± 0.5 (18)	0 (0)	0 (0)
3	26.0 ± 1.5 (59)	25.9 ± 1.5 (18)	$9+3^{2}$ (3)	$6+3^{2}$ (5)
4	50.4 ± 1.6 (28)	50.1 ± 2.0 (16)	$3+6^{2}$ (3)	$1+4^{2}$ (2)
5	74.3 ± 2.1 (9)	75.1 ± 4.2 (6)	1 (4)	$0+4^{2}$ (0)

^{1}Mean ± one standard deviation
^{2}Number of egg masses that were observed + those thought to have been laid because females were no longer gravid

time of the third injection (equal to day 2, Table 5.8), but a substantial proportion of fish that ovulated did so on day 3 (32 %, after three injections), and day 4 (15 %, after four injections, Figure 5.4). Seventy-two percent of all hormonally-injected females that ovulated either tank-spawned or were assumed to have tank-spawned, and 28 % were strip-spawned.
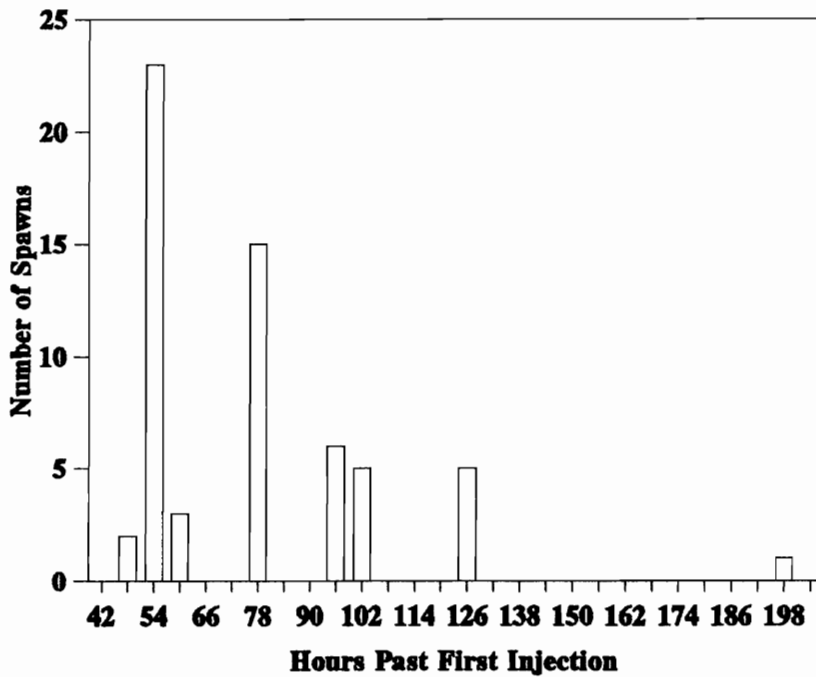
Figure 5.4. Time to spawning of hormonally-injected female <u>Noturus insignis,</u> relative to first injection.

<u>Hormonal Implants</u>

All females had oocytes ≥ 3.3 mm in diameter at the time they received implants, and initial mean oocyte diameters of sham-implanted fish were not statistically different from those of hormonally-implanted fish in either experiment (p-values = 0.15 and 0.34, Table 5.9). Two females that received hormonal implants in the first implant trial died. One had atretic ova at the time of death, and there were only eight large oocytes in the ovaries of the other female. The mean GSI of the remaining hormonally-implanted females was 8.69 ± 1.19 (N = 3), and for sham-implanted females it was 11.74 ± 3.70 (N = 5). These values were not statistically

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Table 5.9. Results of hormonal implant trials with <u>Noturus insignis</u>.

Variable measured	Implant type	
	Sham mean ± SD[1] (N)	Hormonal mean ± SD (N, p)[2]
Male GSI Trial 1	0.60 ± 0.16 (5)	0.64 ± 0.14 (5, 0.65)
Male GSI Trial 2	0.47 ± ---[3] (1)	0.67 ± .06 (3, ---)
Female GSI Trial 1	11.74 ± 3.70 (5)	8.69 ± 1.19 (3, 0.23)
Female GSI Trial 2	3.82 ± 2.31 (3)	8.32 ± 3.71 (3, 0.15)
Initial egg diameter (mm) Trial 1	3.84 ± 0.34 (5)	4.08 ± 0.09 (5, 0.15)
Initial egg diameter (mm) Trial 2	3.40 ± 0.20 (3)	3.53 ± 0.03 (3, 0.34)
Final egg diameter (mm) Trial 1	3.94 ± 0.32 (4)	3.35 ± 0.50 (3, 0.11)
Final egg diameter (mm) Trial 2	2.35 ± 0.69 (3)	3.32 ± 0.98 (3, 0.23)

[1]One standard deviation
[2]Number of fish and probability that mean value differs from that of sham implant
[3]Not determined

different (p-value = 0.23). One of the sham-implanted females had atretic oocytes, and the oocyte diameters of this female were not measured. Mean oocyte diameters for hormonally-implanted fish (3.35 ± 0.50 mm, N = 3) was not significantly different than that of control fish (3.94 ± 0.32 mm, N = 4, p-value = 0.11). All males survived. The mean GSI of hormonally-implanted males (0.64 ± 0.14) was not statistically different than that for sham-implanted males (0.60 ± 0.16, p-value = 0.65). The observed behavior of all fish was considered normal during the experiment. Males and females in each tank utilized the cover afforded by the slab-rock, and they ventured out only after dark or to feed.

In the second trial, two of the three sham-implanted males died. The GSI of the remaining male was 0.47. GSI values for hormonally-implanted fish was 0.67 ± 0.06. All females survived. Sham-implanted females had a mean GSI of 3.82 ± 2.31, and mean final oocyte diameter of 2.35 ± 0.69. Larger vitellogenic oocytes were absent in one of the females. The mean GSI of hormonally-implanted females was 8.32 ± 3.71, and the mean final diameter of oocytes was 3.22 ± 0.98. Oocytes in one of the females were atretic. Neither the final mean egg diameter nor GSI values were significantly different between treatments and controls (p-values = 0.23 and 0.15, respectively).

The fish were checked twice to determine the distribution of fish within each section, and the distribution was the same on both days. In the section with sham-implanted fish, fish were aggregated under two slab-rocks (three fish under each

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rock). In the section with hormonally-implanted fish, a solitary male was observed under one slab-rock, and the other five fish were under another slab-rock. Mean values were not statistically different in either experiment between sham-implanted fish and hormonally-implanted fish for any of the variables measured (Table 5.9).

Hatching Egg Masses

One hundred twelve egg masses were used in trials to develop hatching methods for Noturus spp. Hatching success using incubation Methods 1 through 8 was generally very poor, but occasionally very good hatches were achieved using these methods. For example, 90 % of ova hatched in two egg masses using Method 6, and 62 % of ova hatched in five egg masses using Method 7. However, these egg masses had eyed embryos at the time they were collected. Hatching success for egg masses that did not have visible embryos (the button stage) was always very poor. When sodium sulfite was used to separate eggs in egg masses, premature hatching of embryos occurred. If embryos within egg masses did not hatch within 4 or 5 d, most of them died and turned white. The first successful hatch of eggs that were collected at the button stage occurred when using Method 10 (five larvae hatched from two egg masses). Eighty percent of the eggs of six egg masses that contained more advanced embryos hatched using Method 10. The most consistent high hatch rates were obtained using incubation Method 9. Sixty-eight to 92 % of eggs in 19 of 21 egg masses hatched using this method. Some were at the button stage. Two other egg

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masses at the button stage exhibited relatively poor hatch success using Method 9 (15 and 25 %). One of 2 tank-spawned egg masses incubated using Method 11 did not develop embryos. Sixteen larvae ($\approx$ 35 %) hatched from the other egg mass.

The time to hatch of five egg masses collected at the button stage ranged from 6.5 to 7.5 d at mean temperatures of 26 to 30 C. Larvae were hatched from only one tank-spawned egg mass, in 9 d at a mean temperature of 25 C. The mean number of degree(C)-days required for hatching the six egg masses of the margined madtom was estimated to be 205 $\pm$ 14.

Discussion

The behavior of fish exhibited in tank-spawning experiments was considered normal. They fed well in all experiments, and the only apparent stress incurred by fish, other than disease outbreaks was due to the difficulty in capturing fish from the artificial stream. This stress may have adversely affected the ability of fish to spawn in this experiment. The circling pattern exhibited by margined madtoms in the tank-spawning experiments is similar to the 'cycling' behavior reported by Fitzpatrick (1981) for other madtoms held in aquaria. He considered this behavior to be an integral part of the behavioral repertoire of madtoms in captivity.

It is possible that some fish spawned and that egg masses were consumed by the adults, because nests were not checked every day. However, the absence of the caudal embrace (described by Fitzpatrick 1981) in observations is good evidence that few or no tank-spawnings occurred. Bowen (1980) tank-spawned four of nine pairs of brindled madtoms without using hormonal injections. He was unable to repeat this success with two pairs of brindled madtoms in a second trial; both pairs exhibited spawning behavior, including the caudal embrace, but the females eventually resorbed their ova. Many of the females in my experiments began to resorb their oocytes after several weeks, and this has been observed for other captive madtoms (Bowen 1980, Mayden and Walsh 1984). Thus, the environmental conditions used in this study were not conducive to tank-spawning by the margined madtom. Clark (1978) and Fitzpatrick (1981) were unable to spawn the speckled madtom, the slender madtom,

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or the brindled madtom in aquaria without hormonal injections. The failure of all pairs of margined madtoms to tank-spawn in this study, and the inability of other researchers to consistently spawn Noturus spp. in tanks without using high dosages of injected hormones, indicates that this method has low potential for spawning rare fishes.

The dosages of hormones that precipitated spawning in Experiments 2, 3, and 4 are similar to, but higher than those reported for spawning most other ictalurids (Table 5.10). The mean effective dosage were 5256 I.U./kg, 58 mg/kg, and 554 μg/kg, for the hormones hCG, CPE, and LHRHa, respectively. The dosages for hCG and CPE are particularly high, because these hormones were used in combination, and presumably would have a synergistic effect. These hormones may work effectively by themselves or at lower doses, but these treatments were not attempted in these experiments. Busch and Steeby (1990) used LHRHa without concurrent dom injections to spawn channel catfish. Thus, LHRHa alone may be just as effective as the LHRHa + dom treatment.

Factors incorporated into the protocol for spawning other ictalurids that may help increase percentage spawn of madtoms include substantial flow of water through aquaria during spawning experiments and providing a variety of spawning shelters (Table 5.10). Tank-spawnings of ictalurids, without hormonal injections, typically have occurred in relatively large tanks (Table 5.10). Thus, use of large tanks, relative

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Table 5.10. Factors associated with spawning of ictalurids held in captivity (CPE=carp pituitary extract, hCG=human chorionic gonadotropin, LHRH=analogues of lutcinizing hormone releasing hormone, PE=pituitary extracts from several species).

Source	Species	Hormone Injected & Dosage/kg or (Fish)	Total Dosage/kg or (Fish)	Days Between and (Number) of Injections	Time (d) to Spawning (Temperature C)	Spawning Tanks/ Substrates	Ratio (Percent) of Spawns
Ryder 1883	Amciurus catus	None	----	----	NA (NA)	Large Aquarium	1:NA
Wallace 1967	Amciurus mclas	CPE 18-25 mg	90-126 mg	3-4 (5)	22 (23)	340 L Aquaria/Gravel	2:2 (100)
Campbell and Branson 1978	Amciurus mclas	CPE[1] 28-31 mg	150-300[2] mg	1 (≤10)	(≤27)	189 L Aquaria	0:8 (0)
Wallace 1972	Amciurus natalis	CPE 19-24 mg	208-287 mg	4 (11-12)	33-36 (23-25)	340 L Aquaria/Gravel, Stones	2:2 (100)
Smith and Harron 1904	Amciurus ncbulosus	None	----	----	47 (24-27)	320 L Tank/ Gravel	1:NA
Breder 1935	Amciurus ncbulosus	None	----	----	>210 (21-22)	1100 L Aquarium/ Gravel, Stones	2:NA
Clemens and Sneed 1957	Ictalurus punctatus	CPE NA	NA	NA (Several)	NA (21-29)	23-132 L Aquaria/ Nonc, H2O Flow	Only a Few
Sneed and Clemens 1959	Ictalurus punctatus	hCG 45-450 I.U.	140-450 I.U.	0.3-1 (1-7)	1-4 (NA)	19-189 L Aquaria/ H2O Flow	39:NA
Sneed and Clemens 1960	Ictalurus punctatus	PE NA	NA	NA	NA (NA)	Mctal Troughs/Small Barrels, H2O Flow	2:3 (67)
Sneed and Clemens 1960	Ictalurus punctatus	PE NA	NA	NA	NA (NA)	Metal Troughs/ H2O Flow	1:13 (8)
Sneed and Clemens 1960	Ictalurus punctatus	CPE 0.5-3.6 mg	1.4-14.5 mg	0.5-4 (1-5, 3[3])	1-22, 93 % 2-5 (NA)	19-189 L Aquaria/ H2O Flow	74:124[4] (60)
Brauhn 1971	Ictalurus punctatus	CPE 20-30 mg	0-57 mg, 25[3] mg	2 (0-2, 1[3])	2-4 (24-26)	500 L Pens in 3100 L Tank/Screen Shelters, H2O Flow	4:7 (57)

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Table 5.10 continued. Factors associated with spawning of ictalurids held in captivity (CPE=carp pituitary extract, hCG=human chorionic gonadotropin, LHRH=analogues of luteinizing hormone releasing hormone, PE=pituitary extracts from several species).

Source	Species	Hormone Injected & Dosage/kg or (Fish)	Total Dosage/kg or (Fish)	Days Between and (Number) of Injections	Time (d) to Spawning (Temperature C)	Spawning Tanks/ Substrates	Ratio (Percent) of Spawns
Carter and Thomas 1977	Ictalurus punctatus	None	---	---	>30 (NA)	1021 L Pen in Raceway/Cavity in Concrete Pipe	2:4 (50)
Carter and Thomas 1977	Ictalurus punctatus	None	---	---	4-8 (27)	1830 L Circular Tanks/ Cavity in Concrete Pipe, H_2O Flow	6:9 (67)
Busch and Steeby 1990	Ictalurus punctatus	None	---	---	<13 (19-26)	76 L Aquaria/H_2O Flow	9:35 (26)
Busch and Steeby 1990	Ictalurus punctatus	CPE 3.0-4.4 mg	9-31 mg	1 (3-7)	3-7 (19-26)	76 L Aquaria/H_2O Flow	6:12 (50)
Busch and Steeby 1990	Ictalurus punctatus	hCG 660-1760 ug	1760-6600 I.U.	1 (1-8)	3-8 (19-26)	76 L Aquaria/H_2O Flow	19:28 (68)
Busch and Steeby 1990	Ictalurus punctatus	LHRH 50-100 ug	100-600 ug	1-4 (1-6)	3-13 (19-26)	76 L Aquaria	26:34 (76)
Fitzpatrick 1981	Noturus exilis	hCG[1] (150 I.U.)	(900 I.U.)	4 (6)	21 (27)	23 L Aquaria/Gravel, Stones, Cans	1:5 (20)
Simonson 1987	Noturus gilberti	hCG 2000 I.U.	1600-2200 I.U.	8-11 (NA)	(NA)	26 L Pens in Raceway/ PVC Shelters/Gravel	0:29 (0)
Wang and Kernehan 1979	Noturus gyrinus	None	---	---	NA (NA)	Aquarium/Gravel, Stones, Vegetation	2:NA
Mayden and Walsh 1984	Noturus hildebrandi	hCG[1] (50 I.U.)	NA	NA	17-35 (23-26)	68 L Aquaria/Mussel Shells, Gravel, Stones, Cans, Flower Pots	5:8 (63)

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Table 5.10 continued. Factors associated with spawning of ictalurids held in captivity (CPE=carp pituitary extract, hCG=human chorionic gonadotropin, LHRH=analogues of luteinizing hormone releasing hormone, PE=pituitary extracts from several species).

Source	Species	Hormone Injected & Dosage/kg or (Fish)	Total Dosage/kg or (Fish)	Days Between and (Number) of Injections	Time (d) to Spawning (Temperature C)	Spawning Tanks/ Substrates	Ratio (Percent) of Spawns
Clark 1978	Noturus leptacanthus	None	---	---	(NA)	Aquaria	0:4 (0)
Bowen 1980	Noturus miurus	None	---	---	361-420 (22-26)	1080 L Tank or 40 L Aquaria/ Flower Pots, Sand	4:13 (31)
Fitzpatrick 1981	Noturus miurus	hCG[1] (50 I.U.)	(50-250 I.U.)	4 (1-5)	2-22 (27)	23 L Aquaria/Gravel, Stones, Cans	8:11 (63)
Burr and Mayden 1982b	Noturus nocturnus	hCG[1] (50 I.U.)	NA	NA (Several)	32 (NA)	Aquaria	3:NA
Fitzpatrick 1981	Noturus nocturnus	hCG[1] (50 I.U.)	(50-100 I.U.)	4 (1-2)	2-7 (27)	23 L Aquaria/Gravel, Stones, Cans	3:3 (100)
Moss 1981	Noturus placidus	None	---	---	(NA)	Aquaria	0:NA (0)
Fontaine 1944	Pylodictis olivaris	None	---	---	300[2] (NA)	11360 L Aquarium/ Gravel, Stones, Stumps	1:1 (100)
Breder 1935	Pylodictis olivaris	None	---	---	NA (NA)	51000 L Aquarium/ Gravel, Stones	1:1 (100)
Clemens and Sneed 1962	Pylodictis olivaris	CPE 4.4-13.2 mg	18-40 mg	1 (3-4)	3-4 (NA)	208 L Aquaria or Cement Trough	2:NA

[1]Females and males injected, otherwise males not injected
[2]Approximated value
[3]Mean value
[4]Includes some fish injected with pituitary extracts from species other than Cyprinus carpio

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to size of fish, may enhance the percentage of madtoms that can be spawned in captivity.

None of the fish injected with high dosages of hormones from which oocyte samples were taken (Experiment 1) ovulated, even though an assessment of the oocytes indicated that they had matured (germinal vesicle breakdown had occurred) in 81 % of the fish, and many pairs exhibited the causal embrace. In Experiment 4, 50 % of the fish ovulated. Experiment 1 and Experiment 4 were identical except that oocytes were not taken from fish in Experiment 4. This implies that sampling of oocytes, using the methods of this study, interferes with the ovulation process. Markmann and Doroshov (1983) used a catheter to monitor seasonal changes in the oocytes of the channel catfish, and concluded that oocyte development was not adversely impacted. They did not, however, report if females spawned following catheterization. Their catheters were prepared from lengths of clear, flexible polyethylene or vinyl tubing and fitted with an 8 cm section of stiff aquarium airline. The outside diameter of this type of tubing is smaller than that of the tubing used in this study, and may cause less trauma to the oviducts and ovaries.

Seventy-two percent of fish in Experiment 2 spawned, but egg masses were observed in only 50 % of these spawns. Of the egg masses observed, those not removed from nests were eaten by the adults, and of the 11 removed from tanks, fertilization was verified in only five. Embryos did not develop in the others.

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Embryos also did not develop in any of the strip-spawned ova of Experiment 2, 3, or 4. The lack of embryo development in all strip-spawned ova, and in some of the tank-spawned egg masses is indicative of a problem with the fertilization process. Channel catfish can be successfully stripped spawned (Clemens and Sneed 1957), but problems with the production of embryos from other strip-spawned ictalurids have been reported for the brown bullhead, Amerius nebulosus, and the white catfish, Amerius catus (Eycleshymer 1901, Gill 1906). Sneed and Clemens (1960) reported that poorly developed male channel catfish will participate in the spawning act, but are unable to successfully fertilize egg masses. I suspect, based on results of experiments in Chapter 4, that the primary problem lies with lack of fertility of the male.

Only one tank-spawning was observed in Experiment 3 even though 10 of 13 fish ovulated. The egg mass produced in Experiment 3 was the only one in this study that was not located within a spawning shelter. Similar results were observed for Experiment 4 in which four of eight females ovulated, but did not tank-spawn. The brindled madtom and the least madtom have spawned in tanks when multiple pairs were present (Bowen 1980, Mayden and Walsh 1984). However, results of Experiments 3 and 4 (one tank-spawn in tanks with multiple pairs), coupled with the results of Experiment 2 (many tank-spawns in tanks with single pairs), imply that while use of multiple pairs of hormonally-injected fish per tank will not preclude ovulation, it will hinder tank-spawning of the margined madtom, and perhaps other

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madtoms. Thus, isolating single pairs of fish, while not requisite to spawning Noturus spp., will likely maximize the percentage of tank-spawnings that can be obtained.

The tendency for male margined madtoms to eat egg masses is a problem. Egg eating has been reported for other ictalurids (Table 5.11). Bowen (1980) reported hatching of a tank-spawned egg mass guarded by a male brindled madtom, but males consumed three other tank-spawned egg masses. Fitzpatrick (1981) was unable to prevent male slender madtoms, brindled madtoms, or freckled madtoms from eating egg masses spawned in captivity by feeding them large quantities of brine shrimp. All eleven spawns produced by these species were eaten by the parents. It is not likely that feeding nest-guarding males will prevent consumption of egg masses, because they apparently don't feed while incubating egg masses in the wild (Clark 1978, Mayden 1980). Egg eating presumably is not restricted to captive fish, because Blumer (1985) reported that 56 of 98 egg masses of the brown bullhead 'disappeared' one day following disturbance to nests. Thus, disturbance to nests seems to be the primary factor that triggers eating of egg masses by the parents, and removal of egg masses from nests to artificially incubate them may be necessary to ensure a high percentage hatch of larvae from tank-spawned egg masses.

Hatching success was low for most of the incubation methods used in this study. The primary problem was that eggs turned white and died within four or five days from the time they were collected, and consequently, poor hatch rates of

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Table 5.11. Summary of factors associated with the production and early care of ictalurid spawns.

Source	Species	Age or Size at Maturity (Longevity)	Sex Building Nest	Caudal Embrace Observed	Sex Incubating Eggs	Incubation Time (d) Degrees (C)	Yolk Absorbed Time (d) Degrees (C)	Egg Mouthing (Eating)
Ryder 1883	Ameiurus catus	---	---	---	Male	6-8 (NA[1])	5-7 (NA)	---
Fowler 1917	Ameiurus catus	---	Both	---	One or both?	---	---	---
Prather and Swingle 1960	Ameiurus catus	---	---	---	---	6-7 (24-29)	---	---
Miller 1966	Ameiurus catus	3-4 yr	---	---	---	---	---	---
Gill 1906	Ameiurus catus	---	---	---	Male	---	---	---
Menzel 1943	Ameiurus catus	18-20 cm	---	---	---	---	---	---
Wallace 1967	Ameiurus melas	---	Female	Male	Male	---	---	(Yes)
Fowler 1917	Ameiurus melas	---	Both	---	Both	---	---	---
Wallace 1972	Ameiurus natalis	---	Female or Both	Both	Male	---	---	---
Fowler 1917	Ameiurus natalis	---	Both	---	One or Both	---	---	(Yes)
Blumer 1985	Ameiurus nebulosus	---	Both	---	Male or Both	5.6[2], 13[3] (NA)	4.4[2] (NA)	Yes (Yes)
McAtee and Weed 1915	Ameiurus nebulosus	---	---	---	Both	---	---	---
Smith and Harron 1904	Ameiurus nebulosus	---	Both	---	Both	5 (25)	6 (25)	Yes
Breder 1935	Ameiurus nebulosus	---	Female or Both	---	Male or Both	6-10 (21-23)	---	Yes (Yes)
Fowler 1917	Ameiurus nebulosus	---	One or Both	---	One or Both	---	---	---

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Table 5.11 continued. Summary of factors associated with the production and early care of ictalurid spawns.

Source	Species	Age or Size at Maturity (Longevity)	Sex Building Nest	Caudal Embrace Observed	Sex Incubating Eggs	Incubation Time (d) Degrees (C)	Yolk Absorbed Time (d) Degrees (C)	Egg Mouthing (Eating)
Breder 1939	Ameiurus nebulosus	---	---	---	One or Both	7 (21)	---	---
Eycleshymer 1901	Ameiurus nebulosus	---	Both	---	Male	---	---	(Yes)
Titcomb 1920	Ameiurus nebulosus	---	---	---	Both	10-14 (20-22)	---	---
Evermann and Clark, 1920	Ameiurus nebulosus	---	Both	---	---	5 (25)	---	---
Langlois 1954	Ameiurus nebulosus	---	---	---	Both	---	---	---
Nelson 1957	Ictalurus punctatus	---	Male	---	Male	9 (21)	---	(Yes)
Jearld and Brown 1971	Ictalurus punctatus	4 yr (13 yr)	---	---	---	---	---	---
Canfield 1947	Ictalurus punctatus	---	---	---	---	8-10 (24)	---	---
Mobley 1931	Ictalurus punctatus	---	---	---	---	7-9[1]	---	---
Wellborn and Schwedler 1981	Ictalurus punctatus	---	---	---	---	8 (25.6)	---	---
Harlan and Speaker 1956	Ictalurus punctatus	33-41 cm	---	---	---	---	---	---
Appelget and Smith 1951	Ictalurus punctatus	33-38 cm, 5 yr	---	---	---	---	---	---
Brown 1942	Ictalurus punctatus	3 yr	Male	---	Male	---	---	(Yes)
Davis 1959	Ictalurus punctatus	---	Male	---	---	---	---	---
Clemens and Sneed 1957	Ictalurus punctatus	---	Both	Both	Male	6-7 (24-28)	---	Yes

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Table 5.11 continued. Summary of factors associated with the production and early care of ictalurid spawns.

Source	Species	Age or Size at Maturity (Longevity)	Sex Building Nest	Caudal Embrace Observed	Sex Incubating Eggs	Incubation Time (d) Degrees (C)	Yolk Absorbed Time (d) Degrees (C)	Egg Mouthing (Eating)
Sneed and Clemens 1960	Ictalurus punctatus	32 cm	---	---	---	6-7 (24-28)	---	---
Menzel 1943	Ictalurus punctatus	23-25 cm	---	---	---	---	---	---
Lenz 1947	Ictalurus punctatus	---	---	---	Male	7-9 (26.7)	---	---
Toole 1951	Ictalurus punctatus	---	---	---	---	5-10 (NA)	---	(Yes)
Shira 1917a & 1917b	Ictalurus punctatus	---	---	---	---	---	5 (22)	---
Burr and Mayden 1984	Noturus albater	---	---	---	Male	---	---	---
Mayden et al. 1980	Noturus albater	1 yr (3 yr)	---	---	Male	8.2 (25)	7 (25)	---
Dinkins 1982	Noturus baileyi	1-2 yr (3 yr)	---	---	Male	---	6 (NA)	---
Burr and Dimmick 1981	Noturus elegans	---	---	---	Male	11.9 (20)	---	---
Starnes and Starnes 1985	Noturus eleutherus	(5 yr)	---	---	Male	---	6 (24)	---
Vives 1987	Noturus exilis	4.7 cm², 1-2 yr	Male	---	---	---	---	---
Mayden and Burr 1981	Noturus exilis	2 yr (5 yr)	---	---	Male	7.8-8.8 (25)	---	---
Fitzpatrick 1981	Noturus exilis	---	---	Both	Male or Both	---	---	(Yes)
Burr and Mayden 1984	Noturus flavater	2 yr (5 yr)	---	---	Male	---	---	---

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Table 5.11 continued. Summary of factors associated with the production and early care of ictalurid spawns.

Source	Species	Age or Size at Maturity (Longevity)	Sex Building Nest	Caudal Embrace Observed	Sex Incubating Eggs	Incubation Time (d) Degrees (C)	Yolk Absorbed Time (d) Degrees (C)	Egg Mouthing (Eating)
Shute 1984	Noturus flavipinnis	2 yr (3 yr)	---	---	Male	---	---	---
Jenkins and Musick 1979	Noturus flavipinnis	6.5 cm (5 yr)	---	---	---	---	---	---
Gilbert 1953	Noturus flavus	3-4 yr (9 yr)	---	---	---	---	---	---
Greeley 1929	Noturus flavus	---	---	---	Male or Both	---	---	---
Walsh and Burr 1985	Noturus flavus	3-4 yr (6 yr)	---	---	Male	---	7 (25)	---
Scott and Crossman 1973	Noturus flavus	(9 yr)	---	---	---	---	---	---
Jenkins and Musick 1979	Noturus gilberti	2 yr (3 yr)	---	---	---	---	---	---
Wang and Kernehan 1979	Noturus gyrinus	---	---	---	Both	---	---	Yes (Yes)
Mahon 1977	Noturus gyrinus	2 yr (4 yr)	---	---	---	---	---	---
Whiteside and Burr 1986	Noturus gyrinus	2 yr (4 yr)	---	---	---	---	---	---
Mayden and Walsh 1984	Noturus hildebrandi	1 yr (1.5 yr)	---	---	Male	8.0-8.7 (25)	9-10 (25)	---
Bowman 1932 and 1936	Noturus insignis	---	---	---	Male	---	> 8 (21.5)	Yes
Fowler 1917	Noturus insignis	---	---	---	Male	---	---	---

Table 5.11 continued. Summary of factors associated with the production and early care of ictalurid spawns.

Source	Species	Age or Size at Maturity (Longevity)	Sex Building Nest	Caudal Embrace Observed	Sex Incubating Eggs	Incubation Time (d) Degrees (C)	Yolk Absorbed Time (d) Degrees (C)	Egg Mouthing (Eating)
Clugston and Cooper 1960	Noturus insignis	9.8 cm², 2 yr (4 yr)	---	---	---	---	---	---
Clark 1978	Noturus leptacanthus	(3 yr)	Male	---	Male	7-8 (24)	10 (24)	(Yes)
Bowen 1980	Noturus miurus	---	Both	Both	Male	8 (25)	8 (25)	Yes (Yes)
Taylor 1969	Noturus miurus	---	---	---	Male	---	---	---
Hardman 1981	Noturus miurus	---	Male	---	Male	---	---	---
Burr and Mayden 1982a	Noturus miurus	1-2 yr (4 yr)	---	---	Male	7.9-9.0) (25)	12 (25)	Yes
Fitzpatrick 1981	Noturus miurus	---	---	Both	Male or Both	---	---	Yes (Yes)
Burr and Mayden 1982b	Noturus nocturnus	1-2 yr (5 yr)	---	---	Male	5.8-6.7 (25)	15 (25)	(Yes)
Fitzpatrick 1981	Noturus nocturnus	---	---	Both	---	---	---	Yes (Yes)
Moss 1981	Noturus placidus	(3 yr)	---	---	---	---	---	---
Taylor 1969	Noturus stigmosus	---	---	---	Male	---	---	---
Fontaine 1944	Pylodictis olivaris	---	Both	Male	Male	7-8 (NA)	6 (NA)	Yes
Minckley and Deacon 1958	Pylodictis olivaris	46-51 cm, 4-6 yr (8 yr)	---	---	---	---	---	---
Breder 1935	Pylodictis olivaris	---	Both	Both	Male	8 (NA)	---	---

[1]Not available
[2]Mean value
[3]Maximum value
[4]Approximated value

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recently spawned egg masses occurred. The white coloration is similar to that caused by egg rot disease which can occur when hatching ova of channel catfish (Tucker and Robinson 1990). Treatment with chemicals did not prevent egg masses from turning white, nor did it seem to enhance hatch rate.

Only egg masses containing visible embryos at the beginning of an incubation period were hatched successfully using Methods 1 to 8. Most other reports of successful hatching of madtom egg masses are those of egg masses collected from the wild at advanced stages of development: the Ozark madtom, Noturus albater, N = 1, 100 % (Mayden et al. 1980); the brindled madtom, N = 1, 86 % (Burr and Mayden 1982a); the slender madtom, N = 5, 39 to 75 % (Mayden and Burr 1981); the orangefin madtom, Noturus gilberti, N=8, 100 % (Simonson 1987). Others have reported problems with attempting to hatch madtom egg masses collected from the wild (Bowman 1936, Clark 1978, Dinkins 1982, Shute 1984). Clark (1978) and Dinkins (1982) were able to hatch eggs of speckled madtoms and smoky madtoms, Noturus baileyi, from more advanced egg masses collected from the wild, but were unable to hatch eggs of newly laid egg masses.

Burr and Mayden (1982b) were able to hatch 100 % of one tank-spawned egg mass of the freckled madtom, and Mayden and Walsh (1984) were able to hatch some larvae from five tank-spawned egg masses of the least madtom (percent hatch not reported). They used 350 mL culture dishes using an air stone for aeration (the use of an air stone is inferred from other publications by the authors). However, I

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was unable to consistently hatch egg masses of margined madtoms, using similar methods (Methods 1, 5, and 7).

Based on the results of my experiments, strong water flow alone is not adequate to consistently yield high hatches of madtom egg masses; agitation of egg masses is also necessary. Breder (1935) came to a similar conclusion after attempting to hatch egg masses of the brown bullhead. The results of his experiment are repeated here because they mirror those of my experiments. "One of the batches of eggs was removed to the laboratory and the following results obtained: All eggs died in less than twenty-four hours in standing water (at the same temperature). All but the few outermost eggs of a cluster died in a flow of water at least equal to that used in trout culture. Eggs lived and hatched when placed in a flask with an inlet reaching to the bottom and with a flow strong enough to keep them in constant tumbling." Fitzpatrick (1981) stated that Mayden (1980) and Clark (1978) had to "introduce vigorous streams of bubbles into bowls to prevent egg mortality that occurred in still water." I was unable to find passages to that effect in those references. Instead, Mayden (1980) mentions use of 111 mm culture dishes and aeration with air stones, and Clark (1978) mentions use of 4 L plastic containers in which "the water was lightly aerated, but not to the extent that the eggs were tumbled about by the turbulence." Clapp (1929) reported that a uniform flow of water over egg masses of the channel catfish yielded much poorer hatching rates than if the water was pulsed. Sufficient agitation to move the whole spawn, but not enough to throw eggs out of

holding baskets is recommended for hatching eggs of the channel catfish in troughs (Huner and Dupree 1984). A tumbling or rocking action may simulate the jostling of eggs that results from the mouthing of egg masses by guardians males. I observed mouthing of margined madtom egg masses by the guardian male several times in the wild, and this action has been exhibited by many other ictalurids (Table 5.11). Fitzpatrick (1981) speculated that manipulation of the egg masses by parents helps provide aeration across the gelatinous barrier produced by the egg matrix.

The egg matrix of channel catfish spawns can be removed by submersion of the egg mass in a solution of sodium sulfite, and this results in dissociation of the egg mass (Dorman 1986). However, it was very difficult to separate egg masses of margined madtoms using sodium sulfite; primarily because there is less egg matrix between ova of egg masses of margined madtoms than those of channel catfish, and the chorions of the ova were very tightly fused. Submersion in the sodium sulfite solution, long enough to separate egg masses of margined madtoms, resulted in weakened chorions and premature hatching of embryos. Premature hatching of other ictalurid embryos has been reported, primarily as a result of handling egg masses (Shira 1917a, Burr and Dimmick 1981, Shute 1984). Premature hatching always resulted in very poor survival of larvae.

Use of sodium sulfite for several minutes only to remove the egg matrix may facilitate hatching of Noturus spp. egg masses by reducing the amount of substrate available for bacterial growth. The only tank-spawned egg mass from which larvae

were hatched was treated with sodium sulfite long enough to remove the egg matrix and incubated in a hatching jar. The water flow was adjusted to a rate that lightly tumbled the egg masses. Another tank-spawned egg mass treated with the above method persisted for several days, but embryos did not develop.

High percentage hatches were achieved when egg masses were placed in plastic baskets (6 mm bar mesh) and the water temperature was 28 to 30 C. These egg masses were subjected to a strong current created with air stones that were located directly below the baskets. The current was strong enough to keep the egg masses in constant motion. Use of an egg basket with 4.5 mm bar mesh netting was not successful, apparently because larvae were too large to drop through the netting when it was folded. Folding the netting to create the basket decreased the size of the mesh openings. Larvae that hatched in the larger mesh baskets and in the hatching jar were robust and generally exhibited good survival in feeding trials (Chapter 6).

The number of degree-days required to hatch embryos of the margined madtom is similar to that reported for six other species of madtoms (range = 200 to 238 degree(C)-days, Table 5.11). Only the speckled madtom and freckled madtom have substantially shorter incubation periods of 180 and 156 degree(C)-days, respectively (Clark 1978, Mayden and Burr 1982a).

Hormonal implants did not result in spawning of single or multiple pairs of margined madtoms. Differences in dosages used or time of administration of implants may result in more positive response of fish to the treatment. Because this

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technique has proven valuable for other species (Huat 1980, Lee et al. 1986b), and it requires less handling of fish, further studies are needed. It is likely that a protocol that is successful for one species of madtom will be applicable to other species.

Recommendations for Future Work

Injections of hCG + CPE or LHRHa + dom produced a satisfactory percentage of spawns of pairs of margined madtoms captured from the wild. However, I cannot recommend these treatments for rare madtoms, because only 50 % of spawned egg masses were observed, and approximately 50 % of those that were observed did not develop embryos.

I also cannot recommend propagation of rare madtoms by hatching egg masses collected from the wild, even though a high percentage hatch of eggs can be obtained from those that contain relatively advanced embryos. Collection of the egg masses requires disturbance of many nests, several of which may contain freshly laid egg masses; they cannot currently be consistently hatched in captivity, and may be eaten if they are left with the male. Frequent disturbance of nest sites may also interfere with the natural spawning process of the madtoms. Thus, collection of egg masses from the wild has strong potential to adversely impact recruitment of larvae of rare madtoms.

Additional experiments that investigate hormonal therapies to spawn madtoms, different than those used in this study, should be undertaken. Pairs of madtoms

should be isolated in those studies, and each pair should be provided with a tank and spawning shelter similar to those used in this study. Spawning success in these tanks should be compared with that in larger tanks. The effects of a moderately strong current created by vigorous aeration or pumps on spawning success should also be investigated. The hormonal treatments used in this study should be used and compared with other treatments. I recommend using combinations of hCG + CPE and LHRHa + dom at lower dosages (one-half and one-quarter the dosages used in this study), because the dosages used in this study are high relative to those used for spawning other ictalurids. I also recommend using a treatment of only hCG, because it has been used to induce spawning of four species of madtoms at dosages of 50 to 150 I.U. per fish (Fitzpatrick 1981, Mayden and Walsh 1984). Because madtoms apparently require several injections of hormones (several days) to spawn, a primer injection may not be requisite. If it is not requisite it should be discontinued, to reduce the handling of captive fish. The percentage of egg masses that develop embryos relative to hormonal treatments should be recorded.

The effects of catheterization on spawning success of hormonally-injected females should be critically evaluated, because egg diameter and germinal vesicle ratio are good indicators of spawning readiness of female madtoms. Diameters of ≥ 3.5 mm and germinal vesicle ratios ≤ 0.10 are desirable. Use of catheters with a smaller outside diameter than the one used in this study, and the effect of one-time catheterization versus the daily catheterization regime used in this study should be

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investigated. Fish that have been catheterized and those that have not, should be hormonally injected and the percentage of spawns in each group should be compared. The percentage of wild fish that spawn versus that of captive fish which have matured using the methods recommended in Chapter 4 should also be compared.

Nests of hormonally-injected fish should be inspected every three or four hours to minimize eating of egg masses by adults. Egg masses should be removed from nests and incubated using either Method 9 or 11 of this study. These methods have potential for hatching egg masses of madtoms that have been collected from the wild, but I cannot recommend using them for hatching egg masses of rare Noturus spp. until they have been perfected. Control of disease organisms, particularly bacteria, seems to be critical to hatching success. Consequently, equipment used in egg hatching experiments should be sterilized before each trial, and the water used should not be from tanks that contain fish or other potential disease vectors. The robustness of larvae hatched by different methods should be monitored.

Fertility of males, particularly the lack of motile sperm, has proven problematic in this study. The lack of embryonic development of egg masses can be attributed to infertility of males. Consequently, methods to ensure fertility of males should be investigated. Motility of sperm of wild males in spawning condition that have been hormonally injected (with each of the treatments recommended above) should be compared to wild males that have not been hormonally injected. An effort

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should be made to collect males that are guarding recently spawned egg masses, and to analyze (i.e., determine sperm motility, GSI, fluid content) testes of those males.

The use of hormonal implants to develop testes of captive males should also be investigated, because they have proven useful for other fish species, and prolonged hormonal therapy can be administered with one-time handling of fish. Implants of LHRHa, hCG, or CPE that use a base of cholesterol and cellulose should be used. The proportion of cellulose should be varied, because higher proportions of cellulose result in faster release of the hormones (Sherwood et al. 1988). Hormone dosages should also be varied, because use of different dosages may enable detection of trends, such as increases in GSI or number of sperm, that can be used to further refine the manufacture of implants for additional trials.

The use of hormonal implants to prevent absorption of oocytes of madtoms held in captivity, and to promote maturation of oocytes of captive females should be investigated. Maintenance of desirable plasma hormone levels may prevent resorption of oocytes of captive females. Implants may also serve as an effective mechanism to hasten maturation of oocytes to a stage (diameter $\geq$ 3.5 mm, and germinal vesicle ratio $\leq$ 0.10) where hormonal injections can be used to induce tank-spawning.

CHAPTER 6

Rearing Larvae of <u>Noturus</u> <u>insignis</u>

Introduction

Only one prior study has examined techniques to rear larval <u>Noturus</u> spp. (Shute et al. 1990). They were able to rear larvae of the yellowfin madtom, <u>Noturus</u> <u>flavipinnis</u>, and the smoky madtom, <u>Noturus</u> <u>baileyi</u>, to an age of approximately three months on a diet of blackworms, cladocerans, mosquito larvae, and blood worms. Survival from the larval stage ranged from 40 to 46 % for the yellowfin madtom, and from 52 to 94 % for the smoky madtom. Other reports on feeding larval madtoms do not describe or evaluate rearing techniques. These studies entailed rearing of larvae for several days to describe the early development of larvae and fry. For example, Mayden and Burr (1981) report feeding crushed catfish chow to larval slender madtoms, <u>Noturus</u> <u>exilis</u>, but they did not report survival or growth rates of the larvae. The goal of this study was to identify a diet, feeding regime, and a system suitable for rearing large numbers of madtoms from larvae to an advanced fry stage.

Rearing procedures for commercially important ictalurids, particularly the channel catfish, <u>Ictalurus</u> <u>punctatus</u>, are well established. In general, fry held in indoor tanks absorb their yolk sac after 5 to 10 d and then swim to the surface, apparently looking for food (Tucker and Robinson 1990). They are then fed up to

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10 times per day on dry diets (Steinbach 1977, Brunson et al. 1983, Robinson et al. 1989). Survival of fry fed a diet of commercial trout or salmon starter is significantly higher than that of fry fed catfish starter (Brunson et al. 1983, Robinson 1989). This may be related to the high protein requirement (58 %) for swim-up fry (Winfree and Stickney 1984), or the relatively high requirement of pantothenic acid in the diet of swim-up fry (Murai and Andrews 1975, Brunson et al. 1983). Consequently, salmon starter and two other high protein diets for larval fish were used in this study. Shute et al. (1990) used a diet of oligochaetes, cladocerans, and mosquito and chironomid larvae to rear yellowfin madtom larvae. The only live food used in this study was brine shrimp. The criteria used to evaluate the results of experiments were fish growth and survival. Larvae of the margined madtom, Noturus insignis, were used in all trials. Extrapolation of results obtained from this study to Noturus spp. was made where appropriate.

Materials and Methods

Three systems were used to rear larvae in this study. Two used large Living Streams ($\approx$ 500 L) as reservoirs and received a small, steady flow ($\approx$ 1 L per minute) of municipal water (Figure 6.1). The flow of water entered at one end of the tank and passed through a bed of activated charcoal prior to mixing with water in the main portion of the tank. One of these systems used four 38 L aquaria (four-tank system) and the other used six (six-tank system), with a resultant total capacity of $\approx$ 700 L of water in both systems that turned over approximately twice per day. The third system was similar to the four-aquaria system described above except that well water was used. Inputs of heated or unheated well water, directed by a computer-controlled automatic system, were used to regulate temperature. The turnover rate of this system was not determined, but several changes of water occurred daily. Both sources of water were satisfactory for rearing larval fishes (Appendix 1).

Submersible pumps were used to pump water from reservoirs to aquaria, and automatic siphons maintained desired water levels. Water from aquaria was returned to the reservoir. Siphons were sections of 13 mm clear, flexible vinyl tubing that were screened to prevent escapement of larvae. The screens were 20 cm diameter circles of 363 μm bar mesh Nitex netting that were formed around the inlet of the siphons and fastened with wire. This provided a relatively large screening area. Holes in the automatic siphons were positioned to maintain tanks at approximately 90 % full ($\approx$ 34 L). Blue-M submersible heaters maintained constant, elevated temperatures

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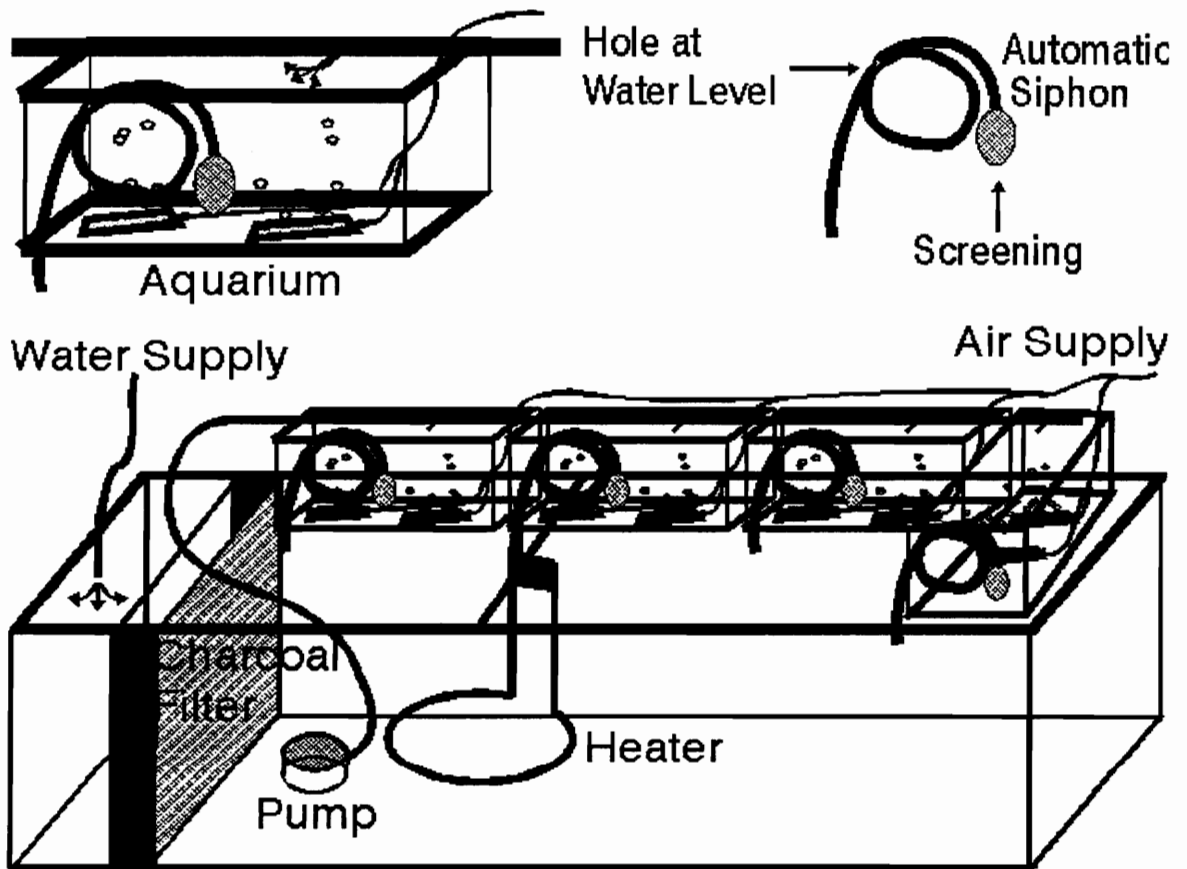
Figure 6.1. Four-tank system used in larval rearing trials.

in the systems that were not regulated by computer control.

Larvae used in all but one rearing experiment were obtained from hatches of margined madtom egg masses collected from local streams. One egg mass was spawned by a hormonally-injected fish in the laboratory. Egg masses were collected from the New River, Montgomery County, Virginia, or its tributary, Wolf Creek in Bland County, Virginia. In the New River, egg masses were found by snorkeling in riffle areas and turning over large slab-rocks. In Wolf Creek, the water was shallow enough that nests could be found by wading and turning over large slab-rocks located in glides. Egg masses were collected with a small, fine mesh aquarium net, or by hand. An effort was made to water buffer egg masses throughout the handling process. They were transported to the laboratory and hatched using methods described in Chapter 5.

Preliminary feeding trials using ground commercial flake food and salmon starter were conducted in which larvae were fed two to four times daily, but survival in these early trials was inconsistent and generally poor. Therefore, only results of the final eight feeding trials are reported herein. They are representative of all feeding trials, and provide the most useful information to develop feeding techniques for Noturus spp.

The transfer procedure consisted of siphoning two to three day-old larvae from hatching tanks using 6 mm I.D. clear, flexible vinyl tubing. The larvae were collected in a 4 L plastic jar with some water to cushion the transfer of larvae from hatching

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tank to collection jar. When the jar filled with water, the water and captured larvae were carefully decanted into aquaria used in feeding trials. Larvae were counted as they were poured from the jar. This process was repeated until all hatched larvae were collected and distributed among rearing tanks. Cover was not provided for larvae because preliminary trials showed that it interfered with cleaning of tanks, and, more importantly, feeding responses of larvae were weaker when cover was present. Larvae seemed reluctant to leave cover to feed.

It is possible that less vigorous larvae were captured at the beginning of a siphoning, and that larval vigor varied among spawns. Consequently, a conscious effort was made to equally distribute larvae collected with each siphoning effort, as well as those from different spawns, among all rearing tanks. Larvae were moved 3 to 5 d after the majority had hatched. Their yolk sacs were still large at the time of transfer, and they ranged in length from 10 to 15 mm (mean = 13.1 ± 1.5 mm).

The number of larvae used in trials depended on clutch size, and subsequent hatching success of each clutch. In Trial 1, 50 to 117 larvae were stocked into each aquarium of the four-tank system that received well water. Trials 2 and 3 were run concurrently. In Trial 2, 52 to 66 larvae were stocked into three aquaria of the six-tank system. In Trial 3, 57 larvae were stocked into an aquarium of the six-tank system three days following the transfer of Trial 2 larvae. In Trial 4, each aquarium in the six-tank system received 32 to 59 larvae. In Trial 5, aquaria in the four-tank system that received well water were stocked with 33 to 39 larvae, and in Trial 6,

aquaria in the six-tank system were stocked with 38 to 44 larvae. All aquaria in the four-tank system that received municipal water were stocked with 70 to 80 larvae in Trial 7, and in Trial 8 one aquarium in this system was stocked with 16 larvae. Trials 1, 4, 5, 6, and 7 were run in duplicate; Trial 2 was run in triplicate. Trial 3 and 8 were not replicated due to a shortage of larvae, but results from the trials are included because they aided in the overall evaluation of rearing techniques. To ensure that replicate tanks were not side-by-side, treatments were randomly assigned to aquaria in consecutive order, and that order was repeated for duplicate tanks. For example, in Trial 4 dry food was fed to fish in tank 1, brine shrimp was fed to fish in tank 2, and a combination of the diets was fed to fish in tank 3. This order was repeated for tanks 4 to 6.

Trials were run at temperatures between 24.0 to 28.5 C. The photoperiod was 16 h light and 8 h dark. Intensity of lighting was gradually changed over a period of at least one-half hour at the beginning and end of each day to preclude startling of larvae. Changes in light intensity were controlled by several timers and incandescent lights or by a computer-controlled dimmer and incandescent lights.

Preliminary results of efforts to develop and maintain cultures of chironomid larvae were unsatisfactory. The production and subsequent supply of these food organisms proved unreliable, and the time required to maintain the cultures was prohibitive. The only live food used in rearing trials was brine shrimp nauplii (Artemia fransicana), because their availability is dependable.

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In preliminary feeding trials, numerous attempts were made to rear larval margined madtoms on a diet of commercial flake food or salmon starter. Poor survival (frequently 0 %) resulted in all trials. Even larvae that seemed to be robust died. Larvae would begin to die relatively soon after feeding was initiated, and then the number surviving gradually dwindled until few or none remained. It was not until rearing tanks were thoroughly cleaned twice daily that survival of larvae approached acceptable levels. Trial 1 is the first trial that employed this procedure, and all other trials reported in this study used the same cleaning procedure.

The dry diets used in feeding trials were salmon starter #0 (420 to 595 μm) and AP100 (350 μm) from Zeigler Brothers, Inc., and Kyowa Fry Feed (400 μm) from Biokyowa Inc. Weight of food for each tank was not premeasured; instead, all tanks were given approximately equal amounts from a container in which the weight of food was known. The mean weight of food per tank per day during each trial was subsequently determined by dividing the total weight of food during a trial by the number of days in the trial and the number of aquaria that received dry food. Fish were fed by hand once in the morning and once in the evening in all feeding trials.

One and six-tenths grams of brine shrimp cysts per tank that received brine shrimp nauplii (3.2 or 6.4 g total) were incubated each day at $\approx$ 27 C in a 3 % NaCl solution. The incubation vessels were inverted 1 L plastic beverage containers without bottoms. They were supported by 500 mL beakers in a water bath that was heated with two 100 watt submersible heaters. Water in the bottles was vigorously

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circulated with a flow of air from small air stones. Incubation was started at the time of the morning feeding. Nauplii were harvested by removing the air stone, allowing settlement to the bottom (cysts to rise to the top) for approximately five minutes, and then siphoning nauplii from the bottom of the bottle with a section of 6 mm I.D. clear, flexible vinyl tubing. Concentrated nauplii were diluted to 800 mL, swirled to uniformly suspend nauplii in the container, and equal volumes were added to each aquarium that received brine shrimp. Approximately one-third of the volume of brine solution in hatching bottles was siphoned and fed in the morning, and the remainder was used for an evening feeding. Thus, the incubation time was 48 or 60 h, and three hatching bottles were required. The maximum density of larvae fed per tank per feeding was estimated to be 5 nauplii per milliliter. For example, if two (four) tanks received brine shrimp, then the number of nauplii per milliliter = 3.2 (6.4) g * 250,000 cysts per gram * 90 % hatch rate / 4 (8) feedings / 34,200 mL = 5.3 nauplii per milliliter.

In preliminary experiments, aquaria were siphoned each day before the evening feeding, but this procedure, as mentioned previously, was not adequate. The cleaning procedure used in this study entailed twice daily siphoning followed by removal of the organic film that developed on tank surfaces within days after feeding was initiated. The organic film was removed by rubbing fingers or a paper towel along the sides and bottom of the tank. The number of dead larvae recovered at the time of each cleaning was recorded. Screens were cleaned as necessary.

The number of larvae that remained after 17 to 28 d was determined, and the lengths of all fry were measured to the nearest 1.0 mm. Percent daily survival was calculated for all trials. Mean lengths of fry in each aquarium were calculated, and the proportion of larvae that survived was determined by dividing the number recovered by the number stocked at the beginning of the trial. Mean length values minus the average size of larvae at the beginning of the trials (13.1 mm) were divided by the number of days in a trial to yield growth per day. Survival rates were not adjusted for number of days in each trial, but this information was used to interpret survival success. Growth and survival rates of replicate tanks were then averaged, and these values were used to rank treatments among trials. The rankings were used to compare results among all trials, and to select the most appropriate techniques for rearing larval madtoms.

Several larvae were reared to 317 d of age on salmon starter. Fish from Trial 1 and other fry, approximately one week younger but not used in larval rearing trials, were stocked into the four-tank system with well water. Thirty-five fry were placed into tank 1 and tank 2, 96 into tank 3, and 51 into tank 4. The fry were initially fed salmon starter #0 (420 to 595 μm). Starter #1 (595 to 841 μm) was added to the diet on day 45, and only #1 was fed after day 67. The diet was switched to starter #2 (0.8 to 1.2 mm) on day 94, and to starter #3 (1.2 to 2.0 mm) on day 142. They were fed starter #3 until the end of the trial. Lengths of these fish were measured on day 114; those of fish in tank 3 were measured again after 276 d

and in all tanks on day 317. Fry from Trial 8 also were reared for several days beyond the end of the larval rearing trial. They were fed a variety of food including salmon starter, commercial flake food, AP100, Kyowa, and occasionally brine shrimp nauplii. Lengths of these fish were measured after 75 d.

Results

Mean temperatures of feeding trials ranged from 24.0 C to 28.5 C. Temperatures did not range beyond one degree of the mean over the course of any trial.

In Trials 6 and 7 the diet of larvae fed dry food may have been supplemented inadvertently by feedings of brine shrimp nauplii. Nauplii were not observed in these tanks, but in rearing trials of larval whitetail shiners, Cyprinella galactura, low densities of nauplii were noted in tanks of similarly designed experiments (Chapter 4). In those experiments, nauplii were transported with overflow water from aquaria that received direct feedings of brine shrimp into the reservoir, and were then pumped into all aquaria. Margined madtom larvae did not rise to the surface several days after hatching, as do larvae of the channel catfish (Tucker and Robinson 1990). Consequently, most food was consumed by larvae after it sank to the bottom of the tank, and food was available to larvae for a long period of time. Amount of dry food per feeding ranged from 300 to 430 mg for all trials, except Trial 5. In Trial 2, the mean weight of dry food per feeding was only 120 mg.

Growth of fish in replicate tanks was similar for all treatments in all trials (Figures 6.2, 6.3, and 6.4). The number of fish in each graph represents the total number of fish recovered at the end of each trial, except the second salmon starter tank (Starter 2) in Trial 1. Only 12 of 34 fish that remained were measured in this tank.

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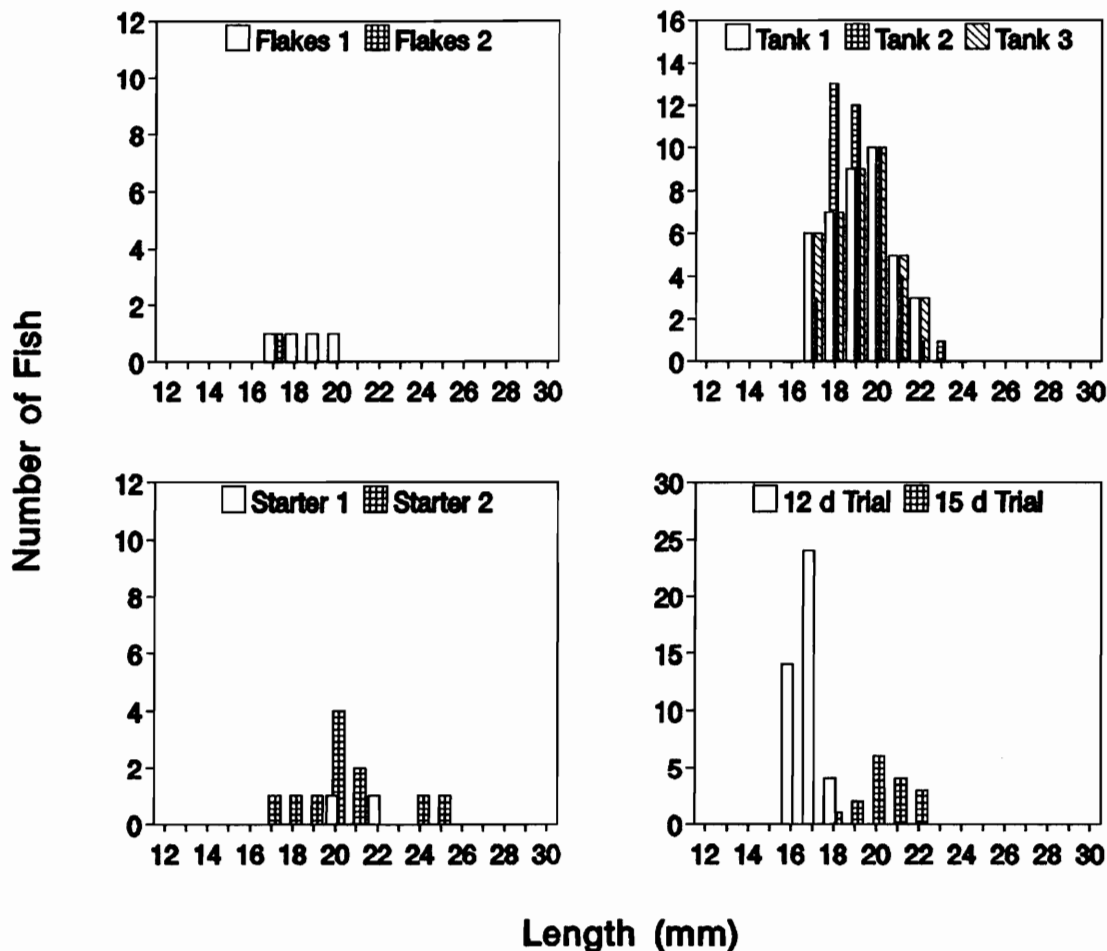
Figure 6.2. Total lengths (mm) of <u>Noturus</u> <u>insignis</u> fry fed flake food and salmon starter in a 28 d feeding trial (left graphs - Trial 1), and salmon starter in 15 d (top right), 12 d, and 15 d (bottom right) feeding trials (Trials 2, 3, and 8).

Growth was also relatively uniform within treatment tanks, as few fry were of a length much larger or smaller than the median value. The length distributions of fish recovered from rearing trials approximated that of a Gaussian distribution in most instances. Peaks in these length frequency distributions occurred between 17 and 22 mm.

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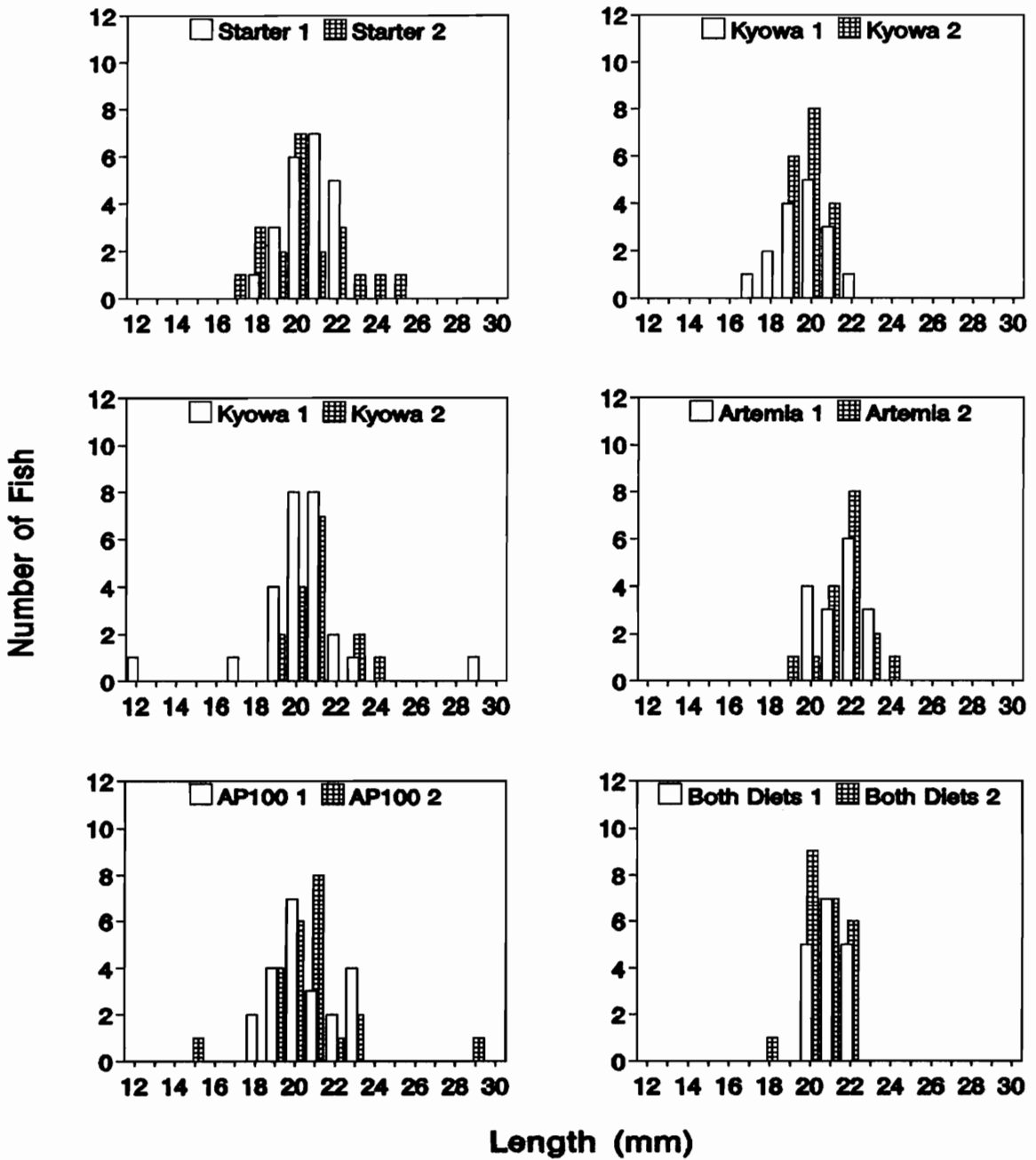
Figure 6.3. Total lengths (mm) of <u>Noturus</u> <u>insignis</u> fry fed salmon starter, Kyowa, or AP100 in a 15 d feeding trial (left graphs - Trial 4), and Kyowa, <u>Artemia</u>, or both diets in a 16 d feeding trial (right graph - Trial 6).
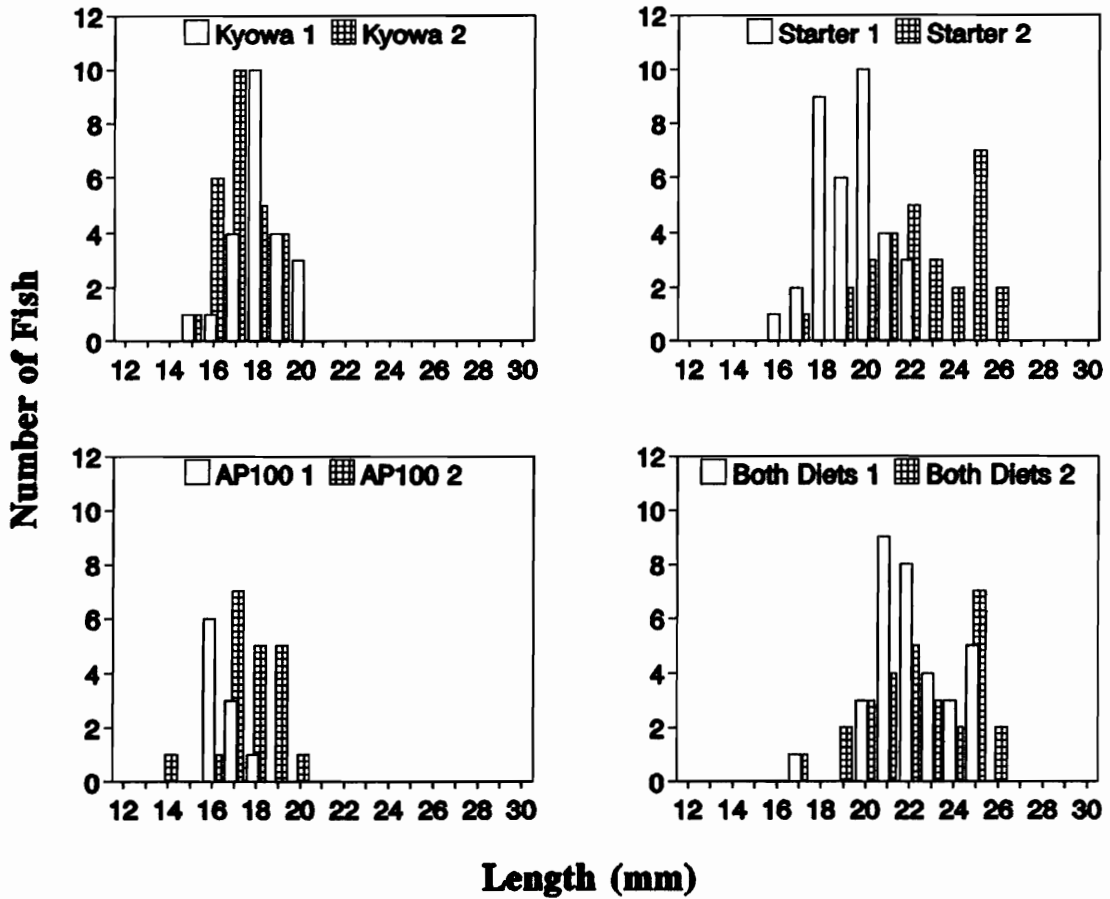
Figure 6.4. Total lengths (mm) of <u>Noturus insignis</u> fry fed AP100 or Kyowa in a 16 d trial feeding trial (left graphs - Trial 5), and salmon starter or salmon starter plus <u>Artemia</u> in a 16 d feeding trial (right graphs - Trial 7).

Mortality of fish in duplicate tanks was also similar for treatments in all trials, and in general it was the same for treatments within a trial (Figures 6.5, 6.6, and 6.7). Exceptions include tanks fed salmon starter in Trial 1, and tanks fed AP100 in Trial 5. The same general pattern of mortality was exhibited in most trials. There was an episode of high mortality that occurred from the time of transfer of larvae to
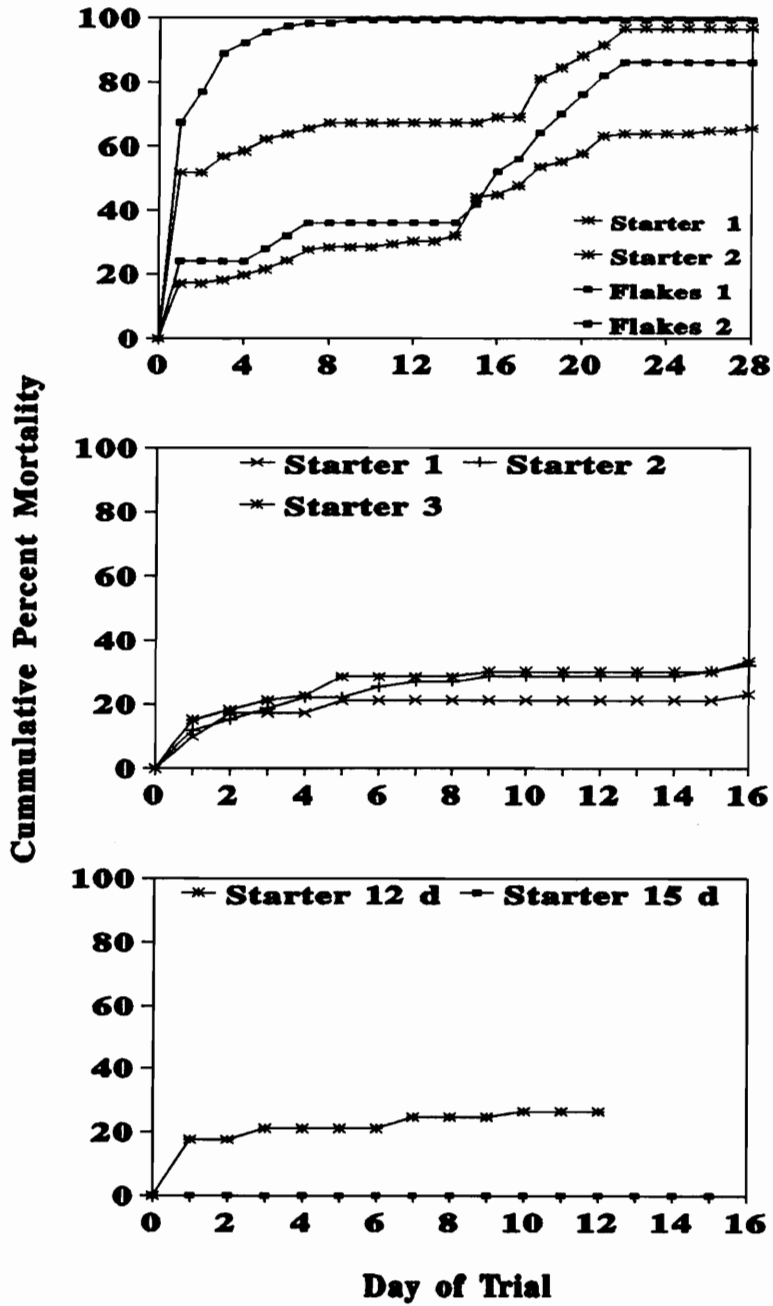
Figure 6.5. Mortality of <u>Noturus</u> <u>insignis</u> fry fed diets of salmon starter or flake food for 28 d (top), salmon starter for 15 d (middle), or salmon starter for 12 or 15 d (bottom) (Trials 1, 2, 3, and 8).
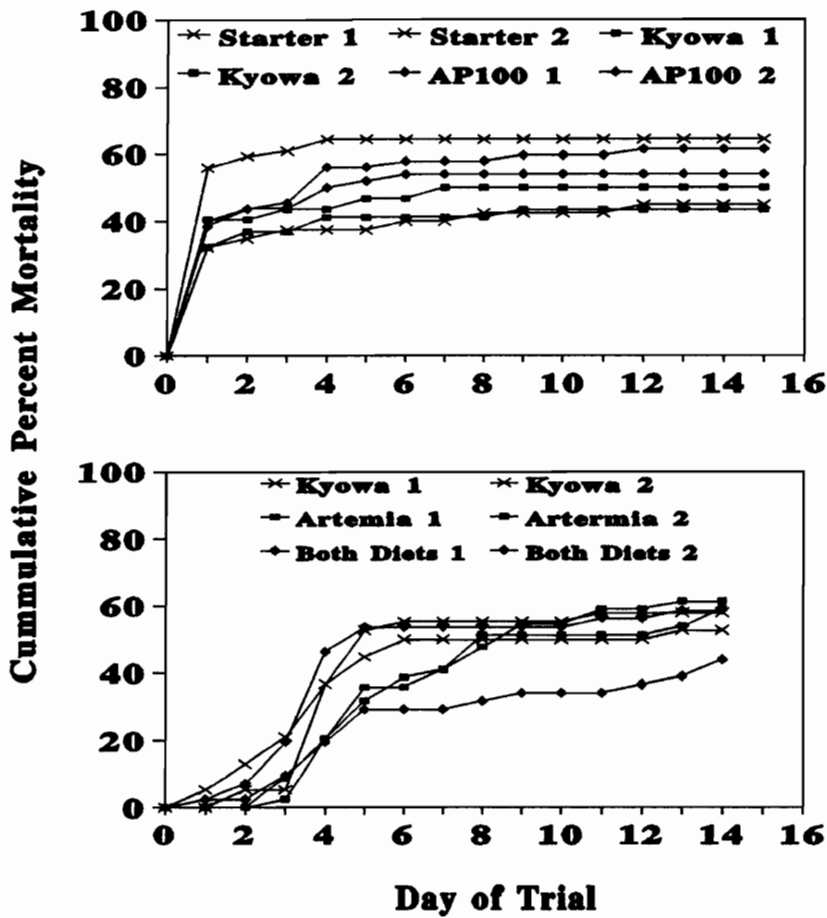
Figure 6.6. Mortality of <u>Noturus insignis</u> fry fed salmon starter, Kyowa, or AP100 in a 15 d feeding trial (top graph - Trial 4), and Kyowa, <u>Artemia</u>, or both diets in a 15 d trial (bottom graph - Trial 6).

approximately day 4. After that time, mortality reached a plateau between 20 and 60 % cumulative mortality for most trials.

Mortality of three tanks in Trial 1 was extremely high (Figure 6.5). This mortality pattern was similar to that observed in most preliminary trials. A mineral deposit formed on the surfaces of tanks used in this trial, as a result of warming the
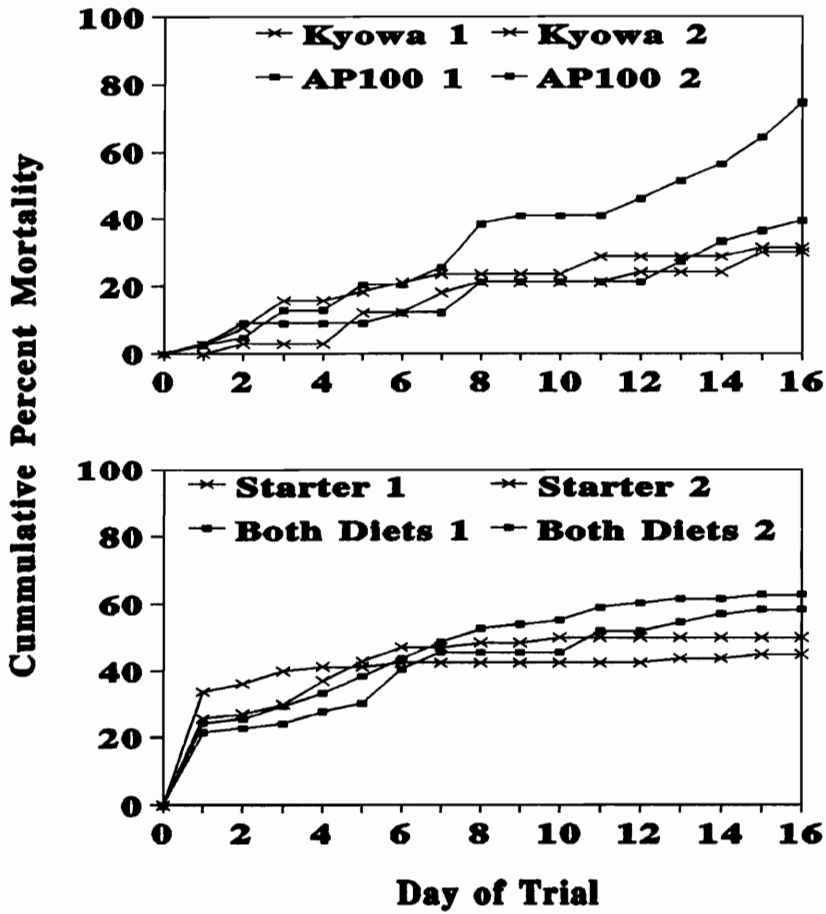
Figure 6.7. Mortality of <u>Noturus</u> <u>insignis</u> fry fed Kyowa or AP100 in a 16 d feeding trial (top - Trial 5), and salmon starter or salmon starter plus <u>Artemia</u> in a 16 d feeding trial (bottom - Trial 7).

well water. The deposit was heaviest in tank one (Starter 1). This same tank had relatively high mortality, when compared to other tanks in Trial 5 (Figure 6.7). Flake food deteriorated more rapidly than salmon starter, resulting in a heavier organic film in those tanks than those that received salmon starter. Greater than 40 % of the mortality that occurred during larval rearing trials for margined madtoms occurred

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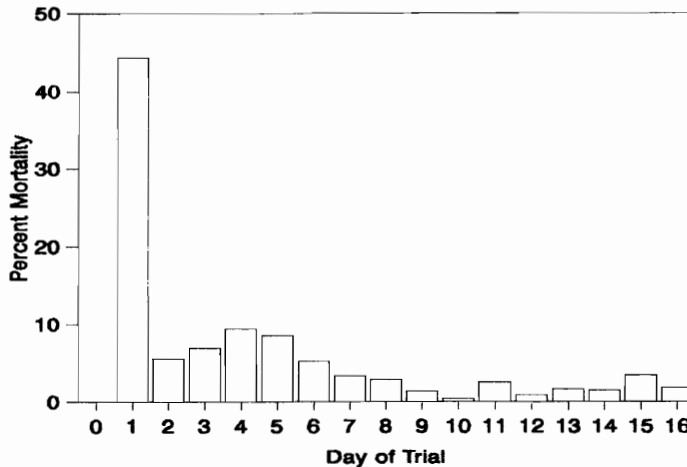
Figure 6.8. Percent of mortality of <u>Noturus</u> <u>insignis</u> fry that occurred by day in all feeding trials combined.

within 2 d, whereas mortality after day 5 was very low (Figure 6.8).

Mean growth rates of margined madtom fry for treatments that had the ten highest growth rates ranged from 1.3 to 1.4 mm per day. The best growth of larval fish was achieved on a diet of brine shrimp, but the survival rate of fish in this trial was one of the lowest (rank = 13 of 15, Table 6.1). The other two treatments that included brine shrimp with a dry diet were ranked 3 and 4 for growth rate, and 7 and 11 for survival rate. The top three survival rates occurred in tanks fed salmon starter. Fish fed diets of Kyowa or AP100 had intermediate growth and survival rates.

The growth of fish reared for 317 d was uniform among tanks on day 114 (64.7 ± 7.7 mm, N=94) and on day 317 (127.3 ± 9.9, N=44, Figure 6.9). Mortality of these fish followed the same general pattern of mortality exhibited in shorter rearing trials (Figure 6.10). There was an episode of high mortality at the beginning of the

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Table 6.1. Comparison of growth and survival of <u>Noturus</u> <u>insignis</u> fry among all feeding trials.

Trial ID length in days (temperature C)	Diet	Amount fed per tank/day (feedings per day)[1]	Mean TL ± SD[2] per tank	Percent survival (number[3] of fish)	Rank G+S[4]
Trial 6 15 (27.0)	Artemia	550 mg dry	21.5 ± 1.1 21.7 ± 1.1	41 (39) 39 (44)	1+13
	Kyowa + Artemia	1.6 mg cysts (2)	21.0 ± 0.8 20.7 ± 1.0	41 (41) 44 (41)	4+11
	Kyowa		19.6 ± 1.3 19.9 ± 0.7	42 (38) 47 (38)	9+9
Trial 3 12 (28.5)	Salmon starter	750 mg (1)	16.8 ± 0.6	74 (57)	2+2
Trial 7 16 (24.5)	Starter + Artemia	860 mg of dry	22.2 ± 1.8 22.5 ± 2.3	42 (79) 50 (78)	3+7
	Salmon starter	0.8 mg cysts (2)	19.3 ± 1.5 20.5 ± 1.5	37 (70) 55 (80)	10+7
Trial 4 15 (25.5)	Kyowa	600 to 760 mg	20.4 ± 2.6 20.9 ± 1.3	57 (46) 50 (32)	5+5
	AP100	(2)	20.5 ± 1.6 20.7 ± 2.4	39 (57) 46 (50)	6+11
	Salmon starter		20.6 ± 1.1 20.4 ± 2.0	55 (40) 49 (59)	7+6
Trial 8 15 (24.0)	Salmon starter	ND[5] (2)	20.4 ± 1.1	100 (16)	8+1
Trial 2 15 (28.5)	Salmon starter	750 mg (1)	19.3 ± 1.5 19.2 ± 1.5 19.1 ± 1.3	77 (52) 68 (59) 67 (66)	11+3
Trial 5 16 (25.3)	Kyowa	240 mg (2)	18.0 ± 1.2 17.2 ± 1.1	70 (33) 68 (38)	12+4
	AP100		16.5 ± 0.7 17.7 ± 1.3	26 (39) 61 (33)	13+10
Trial 1 28 (24.7)	Salmon starter	ND (1 or 2)	21.0 ± 1.0 21.4 ± 1.9	3 (58) 34 (116)	14+14
	Flake food		18.5 ± 1.1 17.0 ± 0	8 (50) 1 (117)	15+15

[1]Weight of cysts is weight of <u>Artemia</u> cysts prior to incubation
[2]Total length (mm) of fish ± one standard deviation
[3]Number of fish at beginning of trial
[4]Ranking of mean growth per day (G) and survival (S) of each treatment over all trials
[5]Not determined, but similar to other trials that used salmon starter

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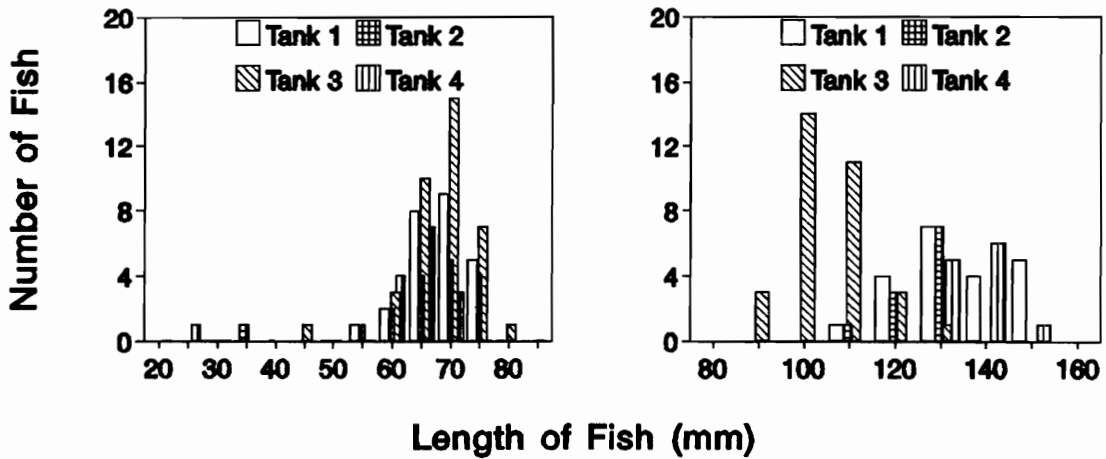
Figure 6.9. Total lengths (mm) of <u>Noturus</u> <u>insignis</u> fingerlings fed salmon starter for 114 d (left graph), and for 276 d (Tank 3) or 317 d (Tanks 1, 2, and 4, right graph).
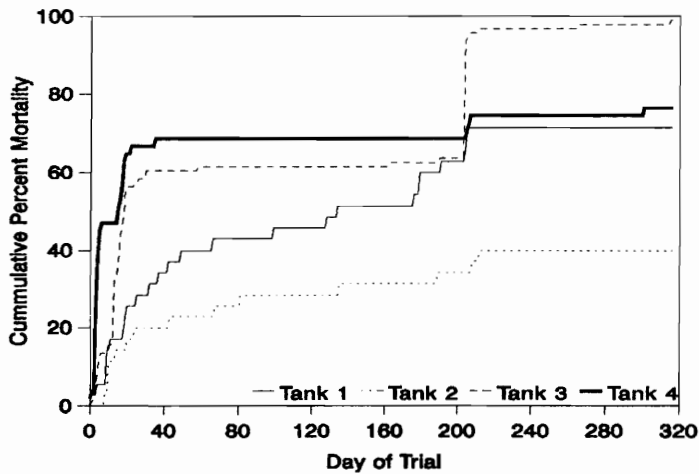
Figure 6.10. Mortality of <u>Noturus</u> <u>insignis</u> fingerlings fed salmon starter for 317 d.

rearing trial, and then mortality plateaued in all tanks except tank 1. In this tank, mortality increased in a stair-step fashion. This was the same tank that was subject to relatively heavy build-up of precipitate from heated well water in Trials 1 and 5. Mortality of fish after day 40 was associated with failure of the temperature-controlling mechanism or pumps. Failure of the temperature controls resulted in

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temperature decreases as great as 10 C. Pump failures, caused by build-up of precipitate in impellers, resulted in smaller changes in temperature, and deterioration of water quality. All but one fish died in tank 3 on day 276 as a result of a pump failure, and a rapid deterioration in water quality commensurate with the high density of fish in the tank at that time. All fry from Trial 8 survived to 90 d of age. The mean length of these fish was 51.9 ± 9.0 mm.

Discussion

Growth and survival of margined madtom larvae was satisfactory on all diets except commercial flake food. Twice daily feedings of a dry diet or brine shrimp nauplii were sufficient to produce good growth and survival. The primary factor that influenced results of margined madtom rearing trials seemingly was the cleanliness of rearing tanks rather than the diets used. The development of an organic film on the surface of tanks was related to poor survival of margined madtom larvae. The perceived need for thoroughly cleaning tanks at least twice daily is reinforced by the poor survival of fish in tank 1 of the four-tank system that used well water (Trial 1 and Trial 5), and by the poor survival of larvae fed a diet of commercial flake food. Tank 1 was subject to a heavier buildup of precipitate from warmed well water, and flake food deteriorated faster than other foods. Twice daily cleanings are required independent of water quality, because water quality was very good even in preliminary trials in which most or all fish died.

Cleanliness of rearing troughs was stressed in early efforts to rear channel catfish fry (Canfield 1947, Morris 1939). Tucker and Robinson (1990) state that ictalurid eggs and fry are very susceptible to disease, but consider once daily removal of organic debris sufficient. Apparently this is not the case for <u>Noturus</u> spp. larvae. Shute et al. (1990) had sporadic and generally poor success rearing yellowfin madtom larvae until they incorporated an elaborate system of filters and sterilizers into their rearing procedure. They used live or fresh frozen foods in their experiments. I

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speculate that the large yolk sac of ictalurid fry is very susceptible to invasion by bacteria, and that the lengthy period between hatch and swim-up stage of madtom larvae, unlike channel catfish larvae, prolongs close contact between the yolk sac and very high concentrations of bacteria. Removal of the organic film from the surface of tanks twice daily seemingly reduced the buildup of bacteria.

The best growth rates of margined madtoms in these feeding trials occurred when brine shrimp were part or all of the diet (three of the top four rankings), but the differences in growth rate among the top eight treatment groups was only 0.08 mm/d. Survival rates of fish in tanks that received Artemia nauplii were approximately the same as other treatments within the same experiment that did not receive brine shrimp. Thus, the additional debris that entered tanks, in conjunction with the feeding of brine shrimp, apparently did not substantially impact tank sanitation.

Salmon starter had the three highest rankings for survival, but survival rankings are difficult to interpret among experiments because most mortality occurred in the first few days and could not be attributed to diet alone. Survival of treatments within experiments was similar for all diets except flake food and AP100. The reason for poor survival of fish fed flake food was discussed earlier. The lower survival of larvae fed AP100 compared to Kyowa in Trial 5 can be attributed to use of tank 1 (and the higher amount of precipitate), but in Trial 4, survival of fish fed AP100 was less than that for fish fed either salmon starter or Kyowa.

Approximately half of the mortalities that occurred during feeding trials occurred within two days of stocking. This implies that either the stocking procedure was stressful to larvae or that some larvae were not robust. Abrasion to larvae during siphoning is a likely cause of injury to larvae. I could not, however, separate the contribution of each potential cause of post-stocking mortality in these trials. In Trial 8, all hatched larvae survived for 90 d, demonstrating that post-stocking mortality can be very low.

The substantial reduction in mortality of larvae after 4 or 5 d implies that the feeding techniques used were adequate for rearing madtom larvae. The maintenance of low mortality for 90 d for one group of fish, and for over 300 d in another group of fish, demonstrated that madtom fry can be successfully reared in captivity to the juvenile stage on a diet of dry food.

The tank systems used to rear larvae in this study proved extremely reliable. The influx of fresh water maintained extremely good water quality throughout all feeding trials even when the highest concentrations of dry food were fed (oxygen > 6 mg/L, ammonia < 0.2 mg/L, and nitrite undetectable). The large reservoir is considered essential because it permits dilution of waste products and allows mixing of fresh water with aged water. A gradual turnover of approximately two water exchanges per day permitted maintenance of an equilibrium of important water chemistry parameters such as temperature and pH; a more rapid exchange of the same turnover volume may not. Placement of aquaria over the reservoir was

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beneficial because overflow of tanks resulting from clogged siphon screens or air locks in siphons drained into the reservoir, and this permits normal functioning of the rest of the system. Air locks can be avoided if air stones are kept away from siphon inlets. Clogged screens can also be easily prevented by replacing screens that have begun to biofoul ($\approx$ 1 week) with new screens. Rearing tanks should be cleaned at least once a day before the last feeding. Removal of waste at this time helps ensure good water quality, and guards against buildup of toxic waste products if a failure of the air supply or water supply systems occurs during the night.

The activated charcoal filter ensured that slugs of chlorine or other undesirable chemicals from municipal water supplies did not enter rearing tanks. It also served to directly remove toxic waste products produced by larvae and decomposition of food. The filter may have a secondary water cleansing function, as a substrate for nitrifying bacteria. The large reservoir and turnover rate of water minimize changes in temperature in the event of a heater malfunction.

The simple brine shrimp hatching system also worked extremely well. Poor hatches sometimes occurred if cysts accumulated below air stones that were off the bottom. Thus, air stones used to circulate water in the hatching bottles must be small enough to extend into the neck of the bottle, and they should be placed in the bottom of the neck to ensure agitation of all cysts and a high hatch rate.

Recommendations for Future Work

A tank system similar to that described in this study could be used to rear larval <u>Noturus</u> spp. because the design is simple, effective, and reliable. Duplicate systems should nonetheless be used for rare madtoms to guard against loss of all fish in the event of a system failure. Duplicate parts to systems, such as pumps and heaters, are not deemed necessary inasmuch as the system can function satisfactorily for ≥ 12 h following a malfunction of one or more components. However, back-ups for all parts (tanks, heaters, pumps, air compressors) should be on hand. No other fish should be held in the system, and it should be isolated from tanks of other fish to prevent transfer of disease organisms. As an added precaution against disease, the system should be sterilized prior to rearing larvae, and equipment used in the rearing process should not be interchanged among systems.

Either salmon starter or Kyowa is suitable for rearing <u>Noturus</u> spp. larvae to an advanced fry stage. Salmon starter is recommended because fry were reared to the juvenile stage on a diet of salmon starter, and this circumvents the possibility of mortality that can result when diets are switched. Twice daily feedings of 250 mg per 38 L tank are recommended because 125 mg feedings of AP100 and Kyowa in Trial 5 resulted in lower growth of margined madtom larvae than in other trials in which more food was used. A supplement of brine shrimp (3 to 5 nauplii per liter) should be used with the salmon starter to help ensure an adequate diet. Tank debris, including the organic film that builds up on tank surfaces, must be removed at least

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twice daily. Cover for larvae should not be provided to facilitate feeding and encourage a strong feeding response.

Several strategies should be evaluated to reduce post-stocking mortality resulting from the transfer procedure. Larvae can be hatched in rearing tanks to avoid transfer of fish, but care must be taken to keep tanks clean of the significant amount of organic debris that results from the hatching process. Larger size tubing may reduce abrasion of larvae, but a better method may be to herd larvae into a flat-bottomed container and then move them to rearing tanks. The chances for abrasion would be minimal, and the larvae would be water buffered throughout the process. Netting of larvae is not recommended. Larvae may be more robust and less subject to injury when they are older and have absorbed most of their yolk sac.

I was reluctant to feed more than twice per day because of the apparent impact of heavy concentrations of bacteria on survival of larval madtoms. More frequent cleanings, commensurate with an increase in number of feedings could alleviate this problem, as could cleaning of tanks within minutes of feeding to remove uneaten food. I would not expect more frequent cleanings to increase mortality of larvae, because madtom larvae are large enough to easily avoid cleaning activities.

The effects of additional feedings on survival and growth of <u>Noturus</u> spp. larvae should be investigated. I recommend using Kyowa or salmon starter, supplemented with brine shrimp as treatment groups. Fifty to 100 fish should be stocked per tank, and they should be fed $\approx$ 250 mg dry food per feeding two, four,

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or eight times per day. Brine shrimp should be fed at a rate of three to five nauplii per milliliter twice daily. Tanks should be cleaned either before each feeding, or approximately 30 min after each feeding.

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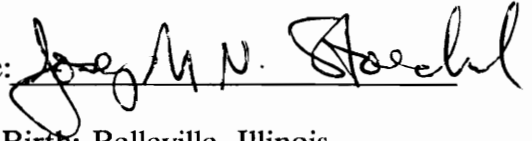
Appendix 1. Analysis of municipal and well water.

Parameter Measured	Municipal Water (mg/L)	Well Water (mg/L)
Arsenic	nd[1]	nd
Barium	nd	nd
Cadmium	nd	0.005
Chromium	nd	0.004
Copper	0.014	0.083
Iron	0.031	0.096
Lead	nd	0.020
Manganese	0.008	0.014
Mercury	nd	nd
Nickel	nd	nd
Selenium	nd	nd
Silver	nd	nd
Sodium	9.5	nd
Zinc	0.004	0.018
Alkalinity (total as $CaCO_3$)	70	270
Chloride	nd	nd
Fluoride	0.6	nd
Nitrate as N	nd	nd
Sulfate	nd	20
Hardness (as $CaCO_3$)	120	370
pH	8.4	7.9
Total Dissolved Solids	165	520
Turbidity	0.1	0.2

[1]Not detectable

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