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A STANDARDIZED ULTRASONOGRAPHY CLASSIFICATION FOR CHANNEL CATFISH OVARIAN DEVELOPMENT

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Renewable Natural Resources

by
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August 2014

To my grandparents and great grandparents, but especially to
my mother, Adela Leocadia Novelo and my father Rodolfo Estevan Novelo,
who dedicated their life to teaching and enriched many lives with
the wonders of hard work, knowledge and wisdom.

To my nieces and nephews, that they may persevere to overcome obstacles, enjoy life,
and achieve their dreams.

And to my friends in Kentucky and in Louisiana, to my extended family and Belizean
community…that we may all be of service to the betterment of humanity
and endeavor to protect the natural resources and beauty of the world.

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Table of Contents

Acknowledgements iii

List of Tables vii

List of Figures xiii

Abstract xxiii

Chapter 1. Dissertation Overview 1
References 6

Chapter 2. Introduction 8
References 16

Chapter 3. A Review of the Use of Ultrasonography in Fish Reproduction 19
Methods 24
Results 25
Discussion 43
References 50

Chapter 4. Development of a Standardized and Systematic Approach to Interpretation of Ovarian Ultrasound Images Captured During Monitoring of Hormone-Induced Spawning of Channel Catfish *Ictalurus punctatus* 56
Methods 59
Results 69
Discussion 88
Summary and Conclusions 93
References 94

Chapter 5. Development, Use, and Evaluation of a Preliminary Ultrasound Imaging Assessment Index for Channel Catfish Ovarian Development 97
Methods 99
Results 108
Discussion 129
Summary and Conclusions 135
References 137

Chapter 6. An Ultrasound Imaging Reference Guide for Channel Catfish Ovarian Development 140
Methods 142
Results 146
Discussion 155
Summary and Conclusions 157
References 158

Chapter 7. Ultrasound Imaging Reproductive Assessment of Channel Catfish Ovaries in Commercial-Scale Hormone-Induced Spawning 160
Methods 163
Results 172
Discussion 180
Summary and Conclusions 183
References 184

Chapter 8. Summary and Conclusions 188
References 198

Appendix A. Standard Operating Procedures 200

Appendix B. Data and Statistics Used in Research Chapters 204

Appendix C. Ultrasonographic Monitoring of Channel Catfish Ovarian Development 225

Appendix D. Letters of Permission 240

Vita 243

List of Tables

1.1 Oral presentations and published abstracts at meetings of the World Aquaculture Society (WAS), the Louisiana Chapter of the American Fisheries Society (LA-AFS), the Southern Division of the American Fisheries Society (SD-AFS), and the Gulf Coast Conservation Biology Symposium (GCCBS) based on dissertation research 2

1.2 Published papers and manuscripts in preparation based on the research presented in this dissertation 3

3.1 Studies of the use of ultrasonography in fish reproduction. The key listing includes the study identification number (ID), the common and scientific names (based on Nelson et al. [2004] whenever possible), the goal of the study, and references. Fish (N = 21 species) were grouped into two main categories—freshwater (24% of species) and marine and anadromous (76%) - according to family, genus, and species. The studies are listed chronologically for each species within a taxonomic order. The goal of each study is identified as sex identification (SI), the development or application of reproductive indices (RI), or both (SI, RI). Sex identification and reproductive indices were divided into two types of data: data based solely on ultrasonography (SI-1, RI-1) and those derived from both ultrasonography and other methods (SI-2, RI-2) 22

3.2 Compilation of data from publications for ultrasound imaging equipment (ultrasound unit and probe), the storage and formatting of images, and the corresponding technical procedures and settings used in fish reproduction studies. The following are reported for the ultrasound units: model, portability, echo display mode used (M), and basic control settings (CS) used for obtaining images (P = power settings [control of voltage, and thus the intensity of sound output by the probe], G = overall gain [causing uniform amplification of all returning echoes regardless of depth of origin], reject settings [causing suppression of returning echoes], and time-gain compensation settings [controlling near to far field amplification of returning echoes]). The probe features include the model, whether it was single or multiple frequency (S/M), the available frequencies (MHz), and the arrangement and shape (array) of the piezoelectric crystal elements, which produced linear (L), annular (A), sector (S), curved (C), and convex sector–linear (CL) image constructs. The data on probe use with regards to frequency transmission procedures and settings included whether the probe was covered, whether it was submersed, what frequency setting (FS) was utilized, and what medium of ultrasound transmission was used (ultrasound transmission gel or water). The ultrasound image storage device (SD) and whether formatting (F) of the ultrasound image was provided were reviewed. (Arr = array; Sub = submersed; Cov = Covered; FU = Frequency used (MHz); R = Reported; NR = not reported) 26

3.3 Compilation of data on fish handling procedures, including the holding system (HS) such as tanks, raceways, or ponds in the laboratory or field, the container used to hold fish during ultrasonography, whether or not the fish was killed before imaging, and whether or not the fish was submersed (Sub) in water, physically restrained (Res), or anesthetized (Anes). Data on fish position (Pos), the external anatomy scanned (scan region), and the duration of the ultrasound imaging procedure (time/fish) are also listed. Yes = reported in the study; NR = not reported in the study 30

3.4 Compilation of biological data on fish species, including the number sampled (No.), the numbers of males (M) and females (F), age (to the nearest year), length (fork [FL], standard [SL], total [TL], or body length to the nearest centimeter), weight (to the nearest kilogram unless specified otherwise), and the terminology used to describe life stage or reproductive condition. These values provided life stage and gonadal maturity data, which were linked to the results of ultrasonography for sex identification and the development of reproductive indices. In general, size was linked to ease of sex identification, and reproductive indices were developed for adult fish during the spawning season or at different stages of gonadal development during the spawning cycle; NR = not reported in the study 38

4.1 All 0.04-ha ponds were stocked with channel catfish broodstock in December, 2004 for the six artificial spawning trials in 2005 listed chronologically according to the date. Four ponds (G10, G9, G8, and G6) were heated in sets of 2 using manipulated geothermal water (36 C) for spawning trials before the start of the natural spawning season. Two ponds (G1, G2) were maintained at ambient water temperature for spawning trials during the natural spawning season. The total weight (kg ± SD) and total length (cm ± SD) of the catfish (n = 12 females/spawning trial) monitored using ultrasonography were listed below 60

4.2 Biological characteristics of males used for artificial fertilization during six spawning trials..... 64

4.3 The ultrasound control settings used for capturing videos of channel catfish ovaries during hormone-induced spawning (brief definitions from the Echo Wave Ultrasound Software Operation Manual, Revision 2.7, 2006). The settings were titled ‘Both Ovaries’ for the gonadal view, and ‘Ovary’ for the gonadal-gametic view. These settings were stored in the pre-set option for documentation, for ensuring consistency in use of ultrasound controls, and for quick retrieval 66

4.4. There were no significant differences ($P > 0.05$) in spawning rates and fertilization among heated or ambient-temperature ponds , the use of all-female or male-female pond stocking (Pond Sex), pairing of fish in male-female or female-female pairings (Tank Pair), sperm concentration, or males used in spawning trials tested. There was no significant difference in spawning latency for all the effects tested, except for Temperature ($P < 0.05$)..... 71

5.1 The sampling date, number of fish, weight and total length (average ± SD), broodstock pond sampled (Pond ID) and the size (ha) were listed for ultrasound imaging of channel catfish ovaries in the recrudescence period during 2008 to 2010 at the LSUAC-ARS in Baton Rouge, Louisiana. Ultrasound images were recorded for fish collected during the seven sampling dates in Windows Bitmap Image format (BMP), and Tagged Image Format Files (TIFF) 100

5.2 Stocking, thermal conditioning, egg mass collection and fish capture by seine for 10 spawning trails during 2008 to 2010. PI = pond identification code; PS = pond size, ha of water; F = Females stocked; M = Males stocked; Date = date of stocking; T = temperature; H = use of geothermal water for heating ponds for reproductive conditioning before the natural spawning season; A = use of ambient temperatures for reproductive conditioning and spawning during the natural spawning season; Egg Masses = egg masses collected from spawning cans..... 101

5.3 Data on fish captured by seine for ovarian ultrasound imaging assessment before ten artificial spawning trails. PI = pond identification code; FC = females collected by seine; FI = The number of females injected with Carp Pituitary Extract in Trial I or Luteinizing Hormone Releasing Hormone analogue in Trials II to X; L = total length (cm) average ± SD; W = weight (Kg) average ± SD. Ultrasound images were recorded for fish collected during the ten spawning trials in the following formats: Audio Video Interleave (AVI), ultrasound image format (USI), Windows Bitmap Image format (BMP), and Tagged Image Format Files (TIFF) 105

5.4 The sampling date, number images recorded, and the time (average ± SD) between consecutive images recorded were listed for ultrasound imaging of channel catfish ovaries in the recrudescence period during 2008 to 2010 at the LSUAC-ARS, Baton Rouge, Louisiana 108

5.5 During the development of the ultrasound imaging classification system preliminary attempts at defining intermediate categories (Other) to microanalyze morphological features between images were discontinued to develop a system in which ovarian development would be easily interpreted and assessed based on the ovarian cycle morphological transformations. 122

5.6 The number of fish injected (Inj.) and the number of fish for which the expected and observed outcomes were the same (Obs.) were listed for three artificial spawning trials in 2008. The percent (%) agreement of the observed and expected outcome (Total Observed/Total Injected x 100) was calculated for seven ultrasound imaging categorical assessments of channel catfish ovarian development. The expected outcome for Categories 1, 2, 3, 6, and 7 was that no viable eggs would be collected, and for Categories 4 and 5 that viable eggs would be collected. The data for categorical assessments 1, 2, 3, 4, 5, 6, and 7 for each trial were used in the statistical analysis comparing the expected to observed outcome 124

5.7 The number of fish injected (Inj.) and the number of fish for which the expected and observed outcomes were the same (Obs.) were listed for four spawning trials in 2009. The percent (%) agreement of the observed and expected outcome (Total Observed/Total Injected x 100) was calculated for seven ultrasound imaging categorical assessments of channel catfish ovarian development. The expected outcome for Categories 1, 2, 3, 6, and 7 was that no viable eggs would be collected, and for Categories 4 and 5 that viable eggs would be collected. The data for categorical assessments 1, 2, 3, 4, 5, 6, and 7 for each trial were used in the statistical analysis comparing the expected to observed outcome 125

5.8 The number of fish injected (Inj.) and the number of fish for which the expected and observed outcomes were the same (Obs.) were listed for four spawning trials in 2010. The percent agreement of the observed and expected outcome (Total Observed/Total Injected x 100) was calculated for seven ultrasound imaging categorical assessments of channel catfish ovarian development. The expected outcome for Categories 1, 2, 3, 6, and 7 was that no viable eggs would be collected, and for Categories 4 and 5 that viable eggs would be collected. All the data for categorical assessments for each trial were used in the statistical analysis comparing the expected to observed outcome..... 126

5.9 Error matrix for ultrasound imaging categories comprising the reproductive development index during 2008-2010. The probability of correct assessment (Mu) was calculated for each expected outcome based on observed outcomes of each ultrasound imaging categorical assessment during the spawning trials. The expected outcome of Categories 1, 2, 3, 6, and 7 was that no viable eggs would be collected. The expected outcome of Categories 4 and 5 was that viable eggs would be collected 127

6.1 Real-time B-Mode ultrasound imaging control settings used for diagnosing ovarian development of channel catfish and for the development of the reproductive classification index (brief definitions from the Echo Wave Ultrasound Software Operation Manual, Revision 2.7, 2006). These settings were stored in the pre-set option of the ultrasound software to ensure consistency in use of ultrasound control settings and for quick retrieval..... 144

6.2 Textual descriptions of the visibility, relative size and echogenic appearance of ovaries for 7 ultrasound imaging categories representative of ovarian development during the channel catfish ovarian cycle 148

6.3 Textual descriptions of the visibility, relative size and echogenic appearance of oocytes for 7 ultrasound imaging categories representative of ovarian development during the channel catfish ovarian cycle 148

6.4 Ultrasonogaphy classification for ovarian development in adult channel catfish 156

7.1 Scanner controls in B-Mode used to assess real-time cross-sectional views of the channel catfish ovary at a commercial-scale were listed below (brief definitions from the Echo Wave Ultrasound Software Operation Manual, Revision 2.7, 2006). These control settings were stored in the pre-set option for ensuring consistency in use of ultrasound controls and for quick retrieval 166

7.2 Ultrasound imaging assessments were based on the visibility, size and echogenic appearance of the ovary along with the oocyte morphology described in Table 7.3, which together defined the seven categories comprising the ultrasound imaging reproductive index 167

7.3 Ultrasound imaging assessments were based on the visibility, size and echogenic appearance of the oocytes along with the morphology of the ovary described in Table 7.2, which together defined the seven categories comprising the ultrasound imaging reproductive index 167

7.4 An equivalency table was developed for standardization of raw data collected during the preliminary development of the ultrasound imaging reproductive index for use in assessment of ovarian development in channel catfish 168

7.5 Dates of ultrasound assessments of channel catfish ovarian development at the Louisiana State University Agricultural Aquaculture Research Station in Baton Rouge, Louisiana (LA) and at Baxter Lands Company hatchery in Arkansas City, Arkansas (ARK) were scheduled based on a preliminary assessment and the two dates selected for transport of fish from LA to ARK for artificial spawning. The sampling identification (ID), the sampling date, the sampling location, and the average time (average ± SD) of ultrasound assessments were listed 169

7.6 Ultrasound assessments during the first set of sampling dates of fish conditioned for reproduction at ambient temperatures at the LSUAC-ARS in Baton Rouge, Louisiana. Of the 860 fish collected by seine from ponds B4 and B6, 313 were assessed as Categories 4 (Advanced) and 5 (Mature). Of the 60 fish assessed at BLC hatchery in Arkansas City, Arkansas using ultrasound imaging after BLC workers had inspected and rejected fish considered not ready for artificial spawning, 32 fish were assessed as Categories 4 (Advanced) and 5 (Mature)..... 175

7.7 Ovarian ultrasound assessments during the second set of sampling dates was of all the fish stocked into two ponds and conditioned for reproduction at ambient temperatures at the LSUAC-ARS in Baton Rouge, Louisiana. Ovarian development of fish conditioned at ambient temperatures in ponds at BLC hatchery in Arkansas City, Arkansas was assessed first by BLC workers based on external morphology. Fish judged as not ready for artificial spawning were returned to the pond. Subsequent ultrasound imaging assessments of ovarian development were of the fish not returned to the ponds 175

7.8 Batch 1 to 5 was comprised of fish conditioned for reproduction at ambient temperatures at the LSUAC-ARS that were selected for transport to BLC hatchery for artificial spawning. Batch 6 was comprised of fish conditioned for reproduction at ambient temperatures at BLC hatchery that were selected for artificial spawning 177

A.1 Ingredients for Hanks' balanced salt solution..... 200

B.1 The equivalency table was used for standardization of the second set of ultrasound imaging sampling dates during 2008 of channel catfish ovarian development in Chapter 7. Ponds B4 (0.3 ha) and B6 (0.3 ha) were at the Louisiana State University Agricultural Center, Aquaculture Research Station in Baton Rouge, Louisiana. Pond 1 (0.4 ha) was at Baxter Lands Company hatchery in Arkansas City, Arkansas 224

B.2 The equivalency table was used for standardization of the second set of ultrasound imaging sampling dates during 2008 of channel catfish ovarian development in Chapter 7. Ponds B4 (0.3 ha) and B6 (0.3 ha) were at the Louisiana State University Agricultural Center, Aquaculture Research Station in Baton Rouge, Louisiana. Pond 1 (0.4 ha), Pond 41 and Pond 3 were at Baxter Lands Company hatchery in Arkansas City, Arkansas. 224

C.1 The species (n = 19) and references (n = 23) on use of ultrasonography in fish reproduction were listed. These fishes were grouped below into two main categories (i) freshwater, and (ii) marine and anadromous, according to family, genus and species, with corresponding citations. It was possible to view ovaries and testes in 85% of the species listed. 227

List of Figures

2.1 Market-sized channel catfish (Photo credit: John Wozniak; Louisiana State University Agricultural Center) 8

2.2 Channel catfish production in the USA in quantity (pounds) for total live weight of food-size fish, broodstock, stockers, fingerling and fry (solid line), and total sales value (dashed line, US $ value) had a steady upward trend from 1988 to 2000, and production remained high until 2006, but began to decline thereafter (Source: USDA NASS 1988 – 2013) 9

2.3 A composite profile of the monthly mean gonadosomatic index of channel catfish and the daily mean ambient air temperature (C) at the Aquaculture Research Station, Louisiana State University Agricultural Station, Baton Rouge, Louisiana. The mean GSI from each study (Trant et al. 1997, Banks et al. 1999) was denoted with different bar colors. The ambient air temperature data was obtained from the Southern Regional Climate Center, Louisiana State University, courtesy of ACIS (Applied Climate Information Systems) and CLIMOD (Climate Information for Management and Operational Decisions)..... 12

2.4 Ultrasound imaging with the probe placed by the left ovary (A), with the near-field view (40 mm depth) of the ultrasound image displayed (B) and the corresponding frozen cross-section of the channel catfish (C) produced images of the main tissue structures including the skin (curved line), muscles, the ovary and the oocytes (encased in the white oval) 14

4.1 Temperature profiles of earthen ponds (0. 04-ha) used for reproductive conditioning of channel catfish broodstock during six spawning trails in 2005. The solid black line represented the ambient temperatures. The threshold temperature of 21 C is indicated by the dashed-dotted straight line. All other lines represent the temperature of heated ponds. Fish captured by seine for each spawning trial are indicated by squares. The sum of the difference of the mean daily temperature and 21 C (degree days) was calculated for quantifying thermal reproductive conditioning from the starting date to the date of capture by seine for each group of fish in heated ponds before the natural spawning period (116, 196, 176, 213 degree days) and for each group of fish in ambient temperature ponds during the natural spawning period (solid black line 192, 303 degree days) 61

4.2 The ultrasound image (bitmap image format) of ovarian cross-sections as they appeared when viewed as gonadal (A) or gonadal-gametic (B) images. The markings on the left of the images indicate intervals of 10 mm depth and the date of still image capture with vestige (i.e. inaccurate) ultrasound controls settings from the still image capture procedure are listed on the right of the image. The white box outlines the area used in figure illustrations. The solid white line demarcates the skin, the dashed line define the ovarian cross-section, the white lines perpendicular to each other within the ovary depict the long (L) and short (S) axes, and the arrow in the white box indicates the direction of ultrasound emission from the probe 67

4.3 Spawning rate (the number of females which produced viable eggs/number of hormone-injected females) indicated by the first bar in each trial (horizontal axis) ranged from 17% (Trial 3) to 40% (Trial 1). The average (±SD) spawning latency (the time from hormone-injection to collection of viable eggs) indicated by the second bar in each trial (horizontal axis) ranged from 32 (±1) (Trial 6) to 57 (±18) h (Trial 2). The average (±SD) neurulation indicated by the third bar in each trail (horizontal axis) ranged from 18 % (±14) (Trail 6) to 73 % (±12) (Trial 1). The use of heated or ambient temperature had a significant effect ($P < 0.05$) on spawning latency (denoted by differences in small letters on the spawning latency bars), but it did not have a significant effect on spawning rates, or fertilization rates 70

4.4 Representative ultrasound images (Fish ID F70) of channel catfish ovaries from which viable eggs were collected. These images were captured using a gonadal view which provided deeper ultrasound penetration but lower resolution, resulting in a broad view of the paired ovaries but no clear image of the oocytes at the time of injection (0 h) up to before (52 h) and after (54 h) egg collection. Differences in the distances of the probe to the skin and the positioning of the ovary resulted from the movement of the free-swimming catfish within the tank of the recirculating system used for artificial spawning. Solid white lines = skin; dashed lines = ovaries; the star indicates the position of the ovary nearest the probe; the arrows point at the areas of ultrasound image artifacts created during fish movement..... 73

4.5 Representative ultrasound images (Fish ID F49) of channel catfish ovaries from which no eggs were collected. These images were captured using a gonadal view which provided deeper ultrasound penetration, lower resolution, a view of the paired ovaries, but no clear image of the oocyte from the time of injection (0 h) up to 80h. Differences in the distances of the probe to the skin and the positioning of the ovary resulted from the movement of the free-swimming catfish within the tank of the recirculating system used for artificial spawning. Solid white lines = skin; dashed lines = ovaries; the star indicates the position of the ovary nearest the probe; the arrows point at the areas of ultrasound image artifacts created during fish movement 74

4.6 Representative ultrasound images (Fish ID F70) of the ovary nearest the probe (denoted by the star in 4.4). These images were captured using a gonadal-gametic view which provided images with less ultrasound penetration but higher resolution, thereby resulting in echogenic appearance of the oocytes encased within the ovary from the time of injection (0h) up to before (52 h) and after (54 h) egg collection. Solid white lines = skin; dashed lines = ovary; the arrows point at the areas of ultrasound image artifacts created during fish movement 75

4.7 Representative ultrasound images (Fish ID F49) of the channel catfish ovary nearest the probe (denoted by the star in 4.5) from which no eggs were collected. These images were captured using a gonadal-gametic view which provided images with higher resolution and the distinct appearance of oocytes encased within the ovary from the time of injection (0h) up to 80h. Differences in the distances of the probe to the skin and the positioning of the ovary resulted from the movement of the free-swimming catfish within the tank of the recirculating system used for artificial spawning. Solid white lines = skin; dashed lines = ovaries; the arrows point at the areas of ultrasound image artifacts created during fish movement..... 76

4.8 Biological (ovals) and ultrasonographic (rectangles) schematic components used for interpretation of ultrasound images of channel catfish ovaries recorded during hormone-induced spawning. Biological insight was informed by biotic and abiotic factors, including the location and tissue components of the ovary, known gonadal processes occurring during artificial spawning (maturation, retention, atresia), water temperature, and observable biological outcomes. The integration of biological and ultrasonographic insights provided a systematic interpretation approach for future development of a standardized classification system (i.e., dashed lines = do not exist at present, and should be the subject for future research). Ultrasonographic insight was informed by use of consistent fish handling and imaging techniques, including the use of the gonadal-gametic view and the size, shape and echogenicity of tissues..... 78

4.9 These still images were captured from the same video for illustration of ultrasound imaging artifacts (arrows) which did not properly represent internal anatomical structures. The airbladder produced hyperechoic reverberation artifacts which appeared as wide white parallel arches nearest the probe with weaker resonating echoes towards the far-field view. These images showed the progression of artifact appearance during image capture from prominent (A, B) to minimized or eliminated (C) so that the ovary was centered in the display image field of view. Water = ultrasound medium, white line = skin, muscle = muscle bundle, and dashed line = periphery of the ovary and oocyte cross-section 81

4.10 Images of oocyte maturation including hydration displayed a visually discernible change in size, shape, and echogenicity. Images of the same fish were recorded at the time of hormone injection (0 h) (left vertical panel) and at the time of egg collection (right vertical panel). Vertical bars of the same size were placed in between the skin (solid white line) and the periphery (dashed line) of the ovary as a visual reference – about 50% reduction of the reference bar in right panel images of enlarged ovaries. Enlarged oocytes were illustrated by same-sized oval white shapes, which encased 4 to 5 oocytes (left panel) and 1 oocyte (right panel). The echogenicity of the ovarian tissues on the left panel provided a global, organized oocyte view, with a light-grey appearance in the center which graduated into a dark-ringed structure in the periphery (oocyte boundary zone. Although hydrated ovary and oocytes retained the characteristic appearance of light-grey in the center and a darker periphery, oocyte boundary zones in some areas of the cross-section were not discernible as the predominant feature of the oocyte center appeared grey, enlarged, and lacked the defined dark-ringed structure of the oocyte periphery, thus imparting a homogenous appearance (triangle area) in the ovarian cross-section 85

4.11 Representative images of ovaries from which no eggs were collected displayed minimal change in size, shape, and echogenicity of ovaries and oocytes. Images on the left vertical panel recorded at the time of hormone injection (0h) were of the same fish (indicated by the fish identification number) as images on the right vertical panel. Vertical bars of the same size were placed in the paired images of the same fish between the skin (solid white line) and the periphery (dashed line) as a visual reference of little or no change in the size of the ovary. The size, shape, and echogenicity of the oocytes in images on the left vertical panel remained similar to images of the right vertical panel – the oocytes appeared with the white-grey center graduating into a darker structure, but no drastic change in size, shape, or echogenicity was present as was distinctly observed for oocytes that underwent oocyte maturation illustrated in Figure 4.10 86

4.12 Representative images of ovaries from which non-viable eggs were observed upon collection as clumpy (F102) and deteriorating (F123) displayed enlarged ovaries of similar size and shape at the time of injection (0 h) and egg collection. However, the shape and echogenicity of the oocytes changed drastically and consequently the echogenic pattern of the ovary changed. Images on the left vertical panel recorded at the time of hormone injection (0 h) were of the same fish (indicated by the fish identification number) as images on the right vertical panel recorded before egg collection. Vertical bars of the same size were placed in the paired images of the same fish between the skin (solid white line) and the periphery (dashed line) of the ovarian cross-section as a visual reference for little or no change in the size of the ovary. However, the shape and echogenicity of the ovary, and in particular, the oocytes, changed. Although oocyte boundaries were visible before egg collection, oocytes appeared irregular in shape, with no distinct center (illustrated by the triangle area) in the majority of the ovary cross-section 87

5.1 Category 1, Undeveloped. The paired horizontal images were randomly selected from ovarian assessments during recrudescence (October – March) on: (A) October 28, 2008 (n = 82 images); (B) November 5, 2008 (n = 89 images); (C) February 3, 2009 (n = 93 images); (D) March 25, 2010 (n = 30 images). The ovaries were visibly identifiable, and the ovarian cross-section appeared as a homogenous echogenic mass with a visible cross-sectional periphery in the near-field view and no visible oocytes. This morphology was best illustrated by ultrasound images in panel A 111

5.2 Category 2, Under-developed. The paired horizontal images were randomly selected from ovarian assessments during recrudescence (October – March) on: (A) October 28, 2008 (n = 10 images); (B) February 3, 2009 (n = 79 images); (C) March 13, 2009 (n = 56 images); (D) March 25, 2010 (n = 25 images). The ovaries were distinctly visible, encasing a heterogeneous echogenic texture within the cross-section which signaled the initiation of oocyte visibility within the image. Although the size of the ovaries varied, the most important echogenic feature was the initial appearance of oocytes, which appeared as specked bright echogenic granules with no shape or form embedded across a darker background, within the ovarian cross-section..... 112

5.3 Category 3, Developing. The paired horizontal images were randomly selected from ovarian assessments during recrudescence (October – March) on: (A) October 28, 2008 (n = 5 images); (B) February 3, 2009 (n = 81 images); (C) March 13, 2009 (n = 55 images); (D) March 25, 2010 (n = 10 images). The ovary was highly discernible, visibly enlarging and appearing closer to skin; oocytes had a higher visibility as denoted by the increased echogenic heterogeneity within the ovarian cross-section; however, oocytes appeared small and compacted within the ovary. 113

5.4 Ultrasound imaging ovarian development of channel catfish collected by seine during the recrudescence period of the reproductive cycle at the Aquaculture Research Station, Baton Rouge, LA. Category 1 (Undeveloped) occurred most frequently in October and November. This ovarian morphology designated as 'Undeveloped' was associated with early vitellogenesis and slow growth period of the ovarian cycle. Category 2 (Under-developed) and 3 (Developing) appeared most frequently in February and March prior to the initiation of the fast growth period of the ovarian cycle. The number of fish assessed = y-axis; the ultrasound imaging categorical assessments = x-axis. 'Other' represented a tentative intermediate categorical assessment of 3.5 during the development of the reproductive index. This attempt at defining a finer spectrum of categorical assessment was abandoned to create a classification assessment index based on easily recognizable biological stages of the ovarian cycle 114

5.5 Category 4, Advanced. The paired horizontal images were randomly selected from ovarian assessments during spawning (April – May) on (A) April 25, 2009 (n = 4 images); (B) May 26, 2009 (n = 8 images); (C) May 5, 2010 (n = 26 images). The ovary appeared prominently within the displayed image, with the ovarian cross-sectional periphery closer to the skin surface and filled with individually distinguishable oocytes, which appeared in an enlarged and organized form throughout the ovary 116

5.6 Category 5, Mature. The paired horizontal images were randomly selected from images recorded during spawning (April – May) on: (A) April 25, 2009 (n = 8 images); (B) May 26, 2009 (n = 25 images); (C) May 17, 2010 (n = 15 images). The ovary appeared extended with the closest proximity to the skin observed, and both ovary and oocytes dominated the displayed image. Oocytes were prominent, large, highly organized, with a discernible boundary 117

5.7 Category 6, Spawned (Spent). The paired horizontal images were randomly selected from images recorded during spawning on: (A) April 25, 2009 (n = 15 images); (B) May 9, 2009 (n = 14 images); (C) June 1, 2009 (n = 11 images). Ovaries appeared small and were barely distinguished against surrounding tissues with few or no oocytes visible. The image denoted by the star shape does not fit this definition and is presented to represent an assessment or data recording error 118

5.8 Category 7, Atretic. The paired horizontal images were randomly selected from images recorded late in the spawning season (May – June) on: (A) May 26, 2009 (n = 81 images); (B) June 1, 2009 (n = 54 images); (C) May 17, 2010 (n = 14 images). These images were of ovaries and oocytes that appeared irregular in echogenic appearance, and irregular and highly variable in shape and size. The double-headed arrow indicates a representative ultrasound image artifact generated by movement of the fish 119

5.9 Ultrasound imaging ovarian development assessments of channel catfish collected by seine from three ponds during 2008 spawning trials. The degree-day (dd) measurements for thermal reproductive conditioning were within (G2, G7 ponds) or greatly exceeded (G6 pond) the window (150 – 172 degree-days) for the conclusion of spawning based on guidelines by Pawiroredjo et al. 2008. Data reported as 'Other' represented tentative intermediate categorical assessments during the earliest part of the development of the reproductive index. "Other' included fish that were assigned intermediate assessments of 3.5 to 4.5 and images in which the ovary was not visible due to ultrasound artifacts caused by visceral fat. This attempt at defining a finer spectrum of categorical assessment was coalesced to create a classification assessment index based on easily recognizable biological stages of the ovarian cycle 120

5.10 Ultrasound imaging ovarian development assessments of channel catfish collected by seine from ponds used in 2009 spawning trials. Ponds R2 and R3 had a reproductive thermal conditioning profile considered to be before the onset of spawning (57 – 81 degree-days). Pond G3 was above the median of the spawning period (99 – 129 degree-days). Ponds G2, B3B, H4 and H7 greatly exceeded the degree-day guidelines defined for the conclusion of spawning (150 – 172 degree-days) and ovarian development assessed as spawned (Category 6) and atretic (Category 7) were at their highest incidence of occurrence in these ponds. Data reported as 'Other' represented tentative intermediate categorical assessments during the development of the reproductive index. "Other' included fish which were assigned intermediate assessments of 3.5 and 4.5 121

5.11 Ultrasound imaging ovarian development assessments of channel catfish collected by seine from ponds used during 2010 spawning trials. The degree-days calculated for ponds G1 and G2 exceeded the window of the conclusion of spawning (150 – 172 degree-days). *Temperature data were not available for two spawning trials 122

6.1 Fish was held in upright swimming position by holding the caudal peduncle with one hand and positioning the probe alongside the fish with the other for ultrasound diagnosis 143

6.2 The position of the probe (indicated by the dashed and solid white lines) was at the top of the image immediately next to the skin (curved line), with the top of the fish (in the area of the dorsal fin) on the right side of the image, and the bottom of the fish (the abdomen area) on the left side of the image. The ovary appeared as if the fish were lying on its side 146

6.3 Category 1. Undeveloped. The ultrasound image (A) represented early and slow growth during the recrudescence phase. The corresponding histological profile (B) of the same ovary in the ultrasound image revealed small oocytes generally less than 1 mm (ruler black bar) characterized by vacuoles (arrow) which do not stain with hematoxylin or eosin appearing along the periphery of the oocyte (Grizzle 1985). Vacuolated oocytes may range from 240 – 650 mm. In addition, oogonia and previtellogenic oocytes which may range from 12 to 120 mm (Grizzle 1985) were observed 149

6.4 Category 2. Under-developed. The ultrasound image (A) represented the first visible sign of vitellogenic activity and occurred early during the recrudescence phase. The corresponding histological profile image (B) of the same ovary in the ultrasound image revealed oocytes characterized by yolk granules (arrow) which filled most of the oocyte cytoplasm 150

6.5 Category 3. Developing. The ultrasound image (A) represented the beginning of fast growth period during the recrudescence phase. The corresponding histological profile (B) of the same ovary in the ultrasound image was observed with high occurrence during February and March during the recrudescence period in Louisiana. The histological profile revealed larger oocytes with and a thin ovarian wall (arrow) 151

6.6 Category 4. Advanced. The ultrasound image (A) represented the fast growth period and advance development during recrudescence. The histological profile (B) of the same ovary in the ultrasound image revealed large oocytes in late vitelline stage; although distinct yolk granules were observed (arrow), the yolk granules were in the process of coallesence (oval shape within the cytoplasm)..... 152

6.7 Category 5. Mature. The ultrasound image (A) represented transition to the spawning period towards the end of the vitellogenesis. The corresponding histological profile (B) of the same ovary in the ultrasound image revealed large oocytes in late vitelline stage that contained coalesced yolk (ovals within the cytoplasm)..... 153

6.8 Spawned. The ultrasound image (A) represented the post spawn or 'spent' state of the ovary in an advanced regressed state. The corresponding histological profile (B) of the same ovary in the ultrasound image revealed a thickened ovarian wall, remnants of atretic oocytes (oval) and oogonia 153

6.9 Category 7. Atretic. The ultrasound image (A) represented visible regressing processes. The corresponding histological profile (B) of the same ovary in the ultrasound image revealed irregular-shaped oocytes (oval) with a thickening ovarian wall (arrow) 154

7.1 The temperature profile (C) for broodstock ponds (0.30-ha) at the Louisiana State University Agriculture Center Aquaculture Research Station in Louisiana (black line) and broodstock ponds (2.55-ha) from which fish originated at Baxter Lands Company hatchery in Arkansas (grey line). The degree-day measure of reproductive readiness for spawning were calculated for the ultrasound imaging sampling dates (dashed line) of ambient water temperatures in Louisiana (the number above the dashed line) and in Arkansas (the number below the dashed line). 173

7.2 Fertilization estimate of egg masses (n = 125 egg masses; 300 – 400 mL eggs/egg mass) that were produced during artificial spawning at BLC hatchery in Arkansas of fish from Batch 1, 2 and 3. These fish were conditioned for reproduction at ambient temperatures at LSUAC-ARS in Louisiana and assessed as Categories 4 (Advanced) and 5 (Mature) of the ultrasound imaging classification index. Egg quality was ≥ 50% fertilization estimate for 39 egg masses (high quality eggs). Egg quality was < 50% fertilization estimate for 85 egg masses (low egg quality) 178

7.3 Fertilization estimate of egg masses (n = 33 egg masses; 300 – 600 mL eggs/egg mass) that were produced during artificial spawning at BLC hatchery in Arkansas of fish from Batch 6. These fish were conditioned for reproduction at ambient temperatures at BLC hatchery in Arkansas and assessed as were comprised of Categories 3 (Developing), 4 (Advanced) and 5 (Mature) of the ultrasound imaging classification index. Egg quality was ≥ 50% fertilization estimate for 4 egg masses (high quality eggs). Egg quality was < 50% fertilization estimate for 29 egg masses (low egg quality) 179

7.4 Ultrasound imaging assessment profile of ovarian development of channel catfish held in broodstock ponds at the LSUAC-ARS in Louisiana 181

8.1 In this hypothetical scenario, using the external examination (manual) process, 60% of fish spawn; 10,000 eggs/fish x 120 fish = 1.2 million eggs/day at 50% survival to fry = 600,000 fry/day x 60 days = 36 Million Fry (Outcome). This same outcome could be achieved by use of ultrasound technology with increased control and efficiency in viable egg collection and use of broodstock resources. The gain would be in the removal of fish that would not produce viable eggs (Categories 3 and 7), and selection of fish that would produce viable eggs (Categories 4 and 5). This would provide different management options to achieve the fry production goal. The first would be to spawn fewer days per year, potentially eliminating days in the first and last weeks of the work season. The second would be to spawn fewer fish per day and obtain the same target within the 60 day work period 195

A.1 Digital images of neurulation was obtained using a stereoscope (Nikon SMZ-U, Tokyo, Japan). A mm-increment ruler was positioned in the upper right corner. Arrows indicate examples of the neurula stage 203

C.1 For viewing, the channel catfish were completely submerged in water, with the ultrasound probe placed on the left side, the cable end (connected to the ultrasound unit) located dorsally (adjacent to the spine), and the probe tip located ventrally (adjacent to bottom of the abdomen) 229

C.2 The image produced by a linear array probe is a rectangular, gray-scale ultrasound image, with the top of the image corresponding to the position of the probe. The cable end of the probe (A) corresponds to the top of the fish (right side of the image), and the tip of the probe (B) corresponds to the bottom of the fish (left side of the image). The top of the image (near field) corresponds to anatomical structures closest to the probe, with the skin depicted by the outermost solid line delineating the curving external perimeter of the fish, followed by muscle tissue, the ovary (dashed curved line), and oocytes (indicated by arrows). The image on the left represents early ovarian development in channel catfish, and the image on the right a more advanced stage 230

C.3 Ultrasonography provided direct images of channel catfish ovaries during the natural spawning season of channel catfish, revealing distinct gonadal appearances for early (A), developing (B, C) and atretic (D) ovaries. The curved solid white line depicts the skin, the anatomical structure closest to the probe. The dashed line depicts the contour of the ovary, with the dorsal and ventral aspect of the fish on the right and left side of each image, and the double-headed arrows between the skin and the ovary showing the changes in thickness of the body wall in each image. Oocytes appear flattened rather than rounded probably due to the polar distributions of aqueous and lipid compartments..... 231

C.4 Histology corresponding to ultrasound images of early (A), developing (B, C) and atretic (D) ovaries of channel catfish during the natural spawning season revealed distinct profiles. Histology corresponding to early (A) ovarian development displayed a thick ovarian wall enclosing numerous small primary oocytes with no discernible wall. Histology of developing ovaries (B, C) displayed large vitelline oocytes with a thin ovarian wall, a visible, thin oocyte wall (B), yolk globule formation (B, dotted oval shapes), a thickened oocyte wall (C), and coalescing yolk globules (C, dotted arrows). Histology of atretic (D) ovaries showed remnants of atretic oocytes, and few small primary oocytes..... 234

Abstract

The goal of this dissertation was to develop application of ultrasonography as a decision-making tool in genetic improvement programs for channel catfish *Ictalurus punctatus*. A literature review on the use of ultrasonography in fish reproduction generated a comprehensive reference data set intended to benefit existing and potential users. It exposed the need for reporting of instrument control settings and standardization of fish handling and imaging procedures. These issues were addressed from the onset of this work by assessing more than 6,300 channel catfish ovaries by use of initial fish handling and imaging procedures developed (2004-2005) at the Louisiana State University Agricultural Center Aquaculture Research Station. The development of a standardized and systematic approach to interpretation of ultrasound images emphasized the interplay of technical and biological aspects of ultrasonography assessments. This showed the importance of the control settings and identified disruptive ultrasound artifacts to avoid for observation of the ovary and oocytes. A preliminary ultrasound imaging classification index for assessing ovarian development during the annual reproductive cycle was developed, used and evaluated. This led to the creation of seven well-defined, standardized ultrasound imaging classifications of channel catfish ovarian development based on the annual cycle. Histology of each ultrasound image in the classification index was included as a Reference Guide to provide insight into the processes observed during ultrasonography. Finally, the ultrasound imaging classification index was used for identification and selection of females for hormone-induced spawning in commercial hatchery production of F_1 hybrids (channel catfish female x blue catfish male *I. furcatus*). In sum, this dissertation provides a systematic method of ultrasound imaging assessment of channel catfish ovarian development enabling progress towards standardization in the use of ultrasonography in fish reproduction.

Chapter 1
Dissertation Overview

The history of ultrasound imaging technology, which uses energy generated by sound waves of 20,000 or more cycles per second, dates back to the discovery of piezoelectricity in 1880 (Gowda et al. 2004). Ultrasound technology was used in the development of patented 'echolocation' sonar devices in 1914 to detect icebergs after the sinking of the Titanic, and it was first used in medical diagnostics to detect brain tumors in 1942 (Kane et al. 2004). It was used to view internal anatomy of submersed human subjects in the 1950s (Walsh et al. 1993). Ultrasound imaging has become a powerful tool that revolutionized farmed animal reproduction research since its introduction in 1980 (Ginther 2014). The use of ultrasonography in fisheries and aquaculture as a reproduction tool in sex identification and development of reproductive indices has been ongoing since the 1980s (reviewed by Novelo and Tiersch in 2012; Chapter 3 of this dissertation) but has not yet become a widely used technique for aquatic species.

This dissertation addresses the developmental pathway and construction of a standardized ultrasound imaging classification index as a decision-making tool in aquaculture reproduction of channel catfish *Ictalurus punctatus*. The use of ultrasonography in channel catfish reproduction provided an innovative approach to ovarian development studies that addressed efforts to increase control and efficiency in reproduction. The findings of these studies have been presented in two peer-reviewed publications, two trade journal articles, one book chapter, and in 17 oral presentations at regional, national and international conferences (Tables 1.1 and 1.2).

Table1.1 Oral presentations and published abstracts at meetings of the World Aquaculture Society (WAS), the Louisiana Chapter of the American Fisheries Society (LA-AFS), the Southern Division of the American Fisheries Society (SD-AFS), and the Gulf Coast Conservation Biology Symposium (GCCBS) based on dissertation research.

Date	Title	Conference	Location
2014	Ultrasonography – A general approach to integrating new technology in research and commercial aquaculture	WAS	Seattle, WA
2013	User's guide to ultrasound imaging in aquaculture reproduction of channel catfish for the laboratory and farm	WAS	Nashville, TN
2012	Formulating an ultrasound imaging classification system for the channel catfish ovarian cycle	WAS[1]	Las Vegas, NV
2012	Ovarian cycle and fisheries management: the channel catfish story	LA-AFS	Baton Rouge, LA
2012	Creative approaches and integration of technologies in addressing the needs of endangered species: ultrasound imaging for channel catfish	GCCBS	New Orleans, LA
2011	Ultrasound practices in fish reproduction	WAS	New Orleans, LA
2011	A review of ultrasound practices in studies of fish reproduction	LA-AFS	LaFayette, LA
2011	Channel catfish as a model for application of ultrasound imaging in aquatic species	GCCBS	New Orleans, LA
2010	Ultrasound imaging of seasonal ovarian development in adult channel catfish	WAS[2]	San Diego, CA
2010	Staging of ovarian development measured by ultrasound imaging and degree days in adult channel catfish	LA-AFS	Baton Rouge, LA
2010	Use of ultrasound imaging to monitor seasonal ovarian changes in adult channel catfish	GCCBS	New Orleans, LA
2009	Ambient conditioning and ultrasound assessment of female channel catfish in Arkansas and Louisiana	WAS	Seattle, WA
2009	The application of ultrasound imaging to gonadal assessment of channel catfish	SD-AFS	New Orleans, LA
2008	Application of ultrasound imaging to gonadal assessment of channel catfish	GCCBS	New Orleans, LA
2008	Evaluation of ultrasound for use in commercial scale induced spawning of catfish	WAS	Lake Buena Vista, FL
2008	Review of ultrasound technology applications for channel catfish in early out-of-season spawning	LA-AFS	Baton Rouge, LA
2007	Use of ultrasound for induced spawning of catfish	GCCBS	New Orleans, LA

[1]Received Runner up Award for Best Student Oral Presentation, [2]Received Best Abstract/Travel Award

Table1.2 Published papers and manuscripts in preparation based on the research presented in this dissertation.

Title	Journal / Book	Status	Chapter
A review of the use of ultrasonography in fish reproduction	*North American Journal of Aquaculture*	Published	3
Fish handling and ultrasound procedures for viewing the ovary of submersed, non-anesthetized, unrestrained channel catfish	*North American Journal of Aquaculture*	Published	-
Ultrasound imaging of channel catfish reproduction	*World Aquaculture*	Published	2*
Ultrasound imaging for use in commercial production of channel catfish	*Louisiana Agriculture*	Published	2*
Ultrasonographic monitoring of channel catfish ovarian development	*Cryopreservation in Aquatic Species*	Published	Appendix C
Development of a systematic approach to interpretation of ovarian ultrasound images captured during monitoring of hormone- induced spawning of channel catfish *Ictalurus punctatus*		In preparation	4
Development, use and evaluation of a preliminary ultrasound imaging assessment index for channel catfish ovarian development		In preparation	5
An ultrasound imaging reference guide for channel catfish ovarian development		In preparation	6
Ultrasound imaging reproductive assessment of channel catfish ovaries in commercial-scale hormone-induced spawning		In preparation	7

*These articles were adapted for use in Chapter 2 of this dissertation with permission of the publishers (Appendix D).

Chapter Overview. Chapter 1 provides the overarching framework and context of the dissertation and describes the role of each chapter. The chapters were organized first by identifying the potential problems in the new use of this imaging technology in channel catfish reproduction. Once these challenges were identified and addressed, the framework was established for the development of a standardized ultrasound imaging classification index.

Chapter 2 provides background information on commercial production of channel catfish, and it identifies the potential benefits of using this technology at commercial hatcheries. This chapter presents the use of ultrasonography to address the need for improvement in reproduction and genetic advancements such as supporting production of the hybrid offspring of channel catfish × blue catfish *Ictalurus furcatus* in commercial hatcheries.

Chapter 3 introduces the central theme of standardization throughout the dissertation. Key to the standardization theme was the observed lack of reporting of control settings used in the ultrasonography studies reviewed. This presents a significant challenge to researchers using ultrasound imaging technology in aquatic animal reproduction. Inadequate or no reporting of settings and imaging procedures limits the use of this technology to niche specialists, minimizes the potential for reproducibility, and handicaps potential new users of this technology in fish reproduction. This need for standardization was first addressed in channel catfish studies at the Louisiana State University Agricultural Center Aquaculture Research Station by the development of rapid, straightforward, consistent fish handling and ultrasound imaging procedures for viewing the ovaries of submersed, nonanesthetized, fish (Guitreau et al. 2012).

These initial procedures provided the starting point for generating the data presented in subsequent chapters of this dissertation.

Chapter 4 addresses how the control settings directly affect the images generated. One group of settings revealed only general ovarian morphology, while other settings used to view the same ovary at the same time revealed detailed structures of the ovary and oocytes. This chapter identifies the control settings that clearly display the ovary and oocyte morphologies that led to the development of a standardized and systematic approach to interpretation of ultrasound images. Chapter 4 also addresses technical aspects of ultrasonography in relation to biological structures and processes observed in the channel catfish, and it proposes the systematization of reporting of figure illustrations to increase understanding and interpretation of ultrasonography images.

Chapter 5 presents the developmental process of creating an ultrasound imaging classification index for use in assessment and selection of fish for artificial spawning. Each of the categories in the ultrasound imaging classification index was evaluated on its relevance to the collection of viable eggs for commercial production based on the agreement accuracy of the hypothesized (expected) and observed outcome in spawning trails during 2008 to 2010. As a result, the strengths, weaknesses, and biological profile of each category were identified.

Chapter 6 presents a reference guide to a well-defined ultrasound classification index developed as a decision-making tool for assessing channel catfish ovaries throughout the reproductive cycle

based on the biological insights and the ultrasonography studies in Chapters 4 and 5 of this dissertation.

Chapter 7 finalizes the transition from laboratory-scale development and use of the ultrasound imaging classification index to its use in commercial-scale identification and selection of females for hormone-induction in the production of the F_1 hybrid from the female channel catfish and male blue catfish.

Chapter 8 presents an overview of the adaptation and development of ultrasound technology for use in channel catfish reproduction. This chapter emphasizes the importance of the mechanisms that will ensure standardization and reproducibility in the use of ultrasonography in channel catfish ovarian reproductive assessment, and in the use of this imaging technology in fisheries and aquaculture applications. It concludes by proposing future directions of integrating ultrasonography into an integrated technology platform for improved reproductive efficiency.

For consistency, the format of the *Journal of the World Aquaculture Society*, with specific modifications to meet Louisiana State University dissertation format and style, was used to prepare this dissertation.

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Chapter 2*
Introduction

Aquaculture is the farming of aquatic animals and plants for local, national and international commerce. The channel catfish *Ictalurus punctatus* (Figure 2.1) has been the most economically important cultured foodfish in the USA, especially in Arkansas, Alabama, Louisiana, Mississippi and Texas, which have been the primary producers (USDA 1988-2013). Channel catfish aquaculture grew from an average annual production of 314,000 lbs (141, 300 kg) in the 1950s to > 500 million lbs (> 225 million kg) between 2000 to 2008, and then declined to 247 million lbs (112 million kg) in 2013 (FAO 2014; USDA 1988-2013). The channel catfish became the most valuable cultured species in the billion-dollar aquaculture sector of the USA through the use of local resources, ingenuity of farmers, and through research by universities and federal and state agencies (Hargreaves and Tucker 2004).



Figure 2.1 Market-sized channel catfish (Photo credit: John Wozniak; Louisiana State University Agricultural Center).

* This chapter was based on two publications which previously appeared as Novelo, N. D. and T. R. Tiersch (2012) 'Ultrasound imaging of channel catfish reproduction' in *World Aquaculture* 43:52-58 and as Novelo, N. D. and T. R. Tiersch (2013) 'Ultrasound imaging for use in commercial production of channel catfish' in *Louisiana Agriculture* 56:14-16. These articles were reprinted in part or in full by permission of the editors of *Louisiana Agriculture* and *World Aquaculture* (Appendix D).

Catfish industry production levels exceeded an annual average of $450 million dollars during 1998 to 2008, and contributed nearly 50% of the total aquaculture sales during 2004 to 2006 (Figure 2.2). After 2006 catfish production began to decline steadily due to major

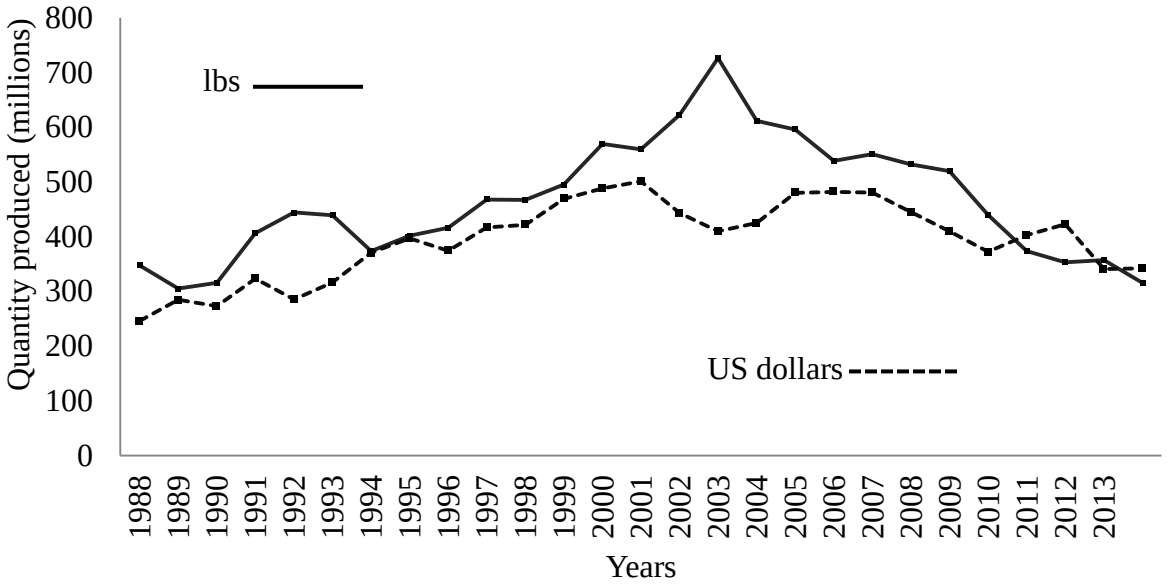


Figure 2.2 Channel catfish production in the USA in quantity (pounds) for total live weight of food-size fish, broodstock, stockers, fingerling and fry (solid line), and total sales value (dashed line, US $ value) had a steady upward trend from 1988 to 2000, and production remained high until 2006, but began to decline thereafter (Source: USDA NASS 1988 – 2013).

economic challenges. These included cheaper imports competing for the well-established catfish market, national recession, high fuel prices, and high prices for corn, soybeans, and other feed ingredients (Anderson et al. 2008). Even prior to the economic challenges and declining catfish production, farms had to find resourceful ways of cutting costs and improving production efficiency while maintaining and increasing profit (Engle and Valderrama 2001; Pomerleau and Engle 2005).

One effective method of improving desirable production traits in aquaculture, as in other fields of agriculture, is through genetic improvement by manipulation of reproductive processes

(Wolters and Tiersch 2004). In the case of the catfish industry in the USA, one rapid means of generating genetic improvement was to produce hybrid catfish obtained from artificial reproduction of the female channel catfish with the male of another closely related species, the blue catfish *Ictalurus furcatus* (C × B hybrid). This hybrid has demonstrated higher returns for feed invested, higher fillet yields, and higher disease resistance than either the channel catfish or blue catfish parents (Giudice 1966; Argue et al. 2003; Li et al. 2004).

The commercial-scale collection of viable channel catfish eggs for fertilization with blue catfish sperm to produce catfish hybrids became necessary because the females and males could not be naturally mated in ponds during the spawning season for commercial production (Perry 1973; Avery et al. 2005; Tave and Smitherman 1982; Wolters and Tiersch 2004). The incorporation of this hybridization technique into the channel catfish industry required new and more specialized steps and it presented a suite of new challenges. These included: (1) handling thousands of individual fish, and distinguishing between males and females, (2) selecting females ready for artificial spawning, (3) purchasing of hormones and anesthetics, (4) hormone injection of females to stimulate egg production, (5) checking for readiness to release eggs, (6) manual collection of eggs, (7) obtaining blue catfish males, (8) killing the blue catfish, (9) obtaining large, fully developed testes, (10) cleaning and crushing the testes to create a sperm solution, (11) development of commercial-scale procedures for manual collection of eggs and fertilization, and (12) investing in labor and training for hybrid catfish production.

These are all indispensable steps in hybrid catfish production; however, one fundamental problematic aspect is egg collection. This requires individual handling of hundreds of female

fish during the spawning season to identify those that are ready for hormone injection and subsequent collection (stripping) of mature eggs. Farmers cull females presumed to have small, undeveloped, immature, or degrading oocytes (eggs). As a result, females with flat bellies traditionally are not considered for artificial spawning efforts, and only females with external features judged to be indicative of readiness for egg collection are selected (Dupree and Huner 1984). Assessing females from external indicators has been used in research and continues to be a method of ovarian assessment to select females for artificial spawning at commercial hatcheries. This external evaluation is highly subjective, varies in practice from farm to farm, and relies heavily on trained workers whose skill level varies among hatcheries (Phelps et al. 2011).

Variable spawning rates have been observed even within natural pond spawning of channel catfish for commercial reproduction, in which only 30-50% of the females in a pond will produce seedstock (Wolters and Tiersch 2004). The selection of females for commercial production of eggs capable of fertilization is a critical component because the ovarian developmental stage at the time of hormone injection directly affects egg quality and dictates the success or failure of spawning induction (Bobe and Labbé 2010; Mylonas et al. 2010). The treatment with hormones to induce oocyte maturation in females will cause the release of poor quality eggs if females with immature oocytes or females with atretic oocytes are injected (Bobe and Labbé 2010; Mylonas et al. 2010; Chatakondi et al. 2011).

Channel catfish undergo an annual ovarian cycle comprised of the recruitment, growth (recrudescence), spawning and resorption phases (Silverstein and Small 2004). The

gonadosomatic index (GSI, i.e. the gonad weight divided by the body weight x 100) was used as a measure of ovarian development during studies of the annual ovarian cycle (Brauhn and McCraren 1975; MacKenzie et al. 1989; Trant et al. 1997; Banks et al. 1999). The GSI profile of channel catfish was largest during the spawning phase, the only phase suitable for selecting fish for hormone-induced spawning, and increased from 8 to 15% in the months of April to June (Figure 2.3). Ovarian development during most of the annual cycle is inadequate for hormone-induced spawning as indicated by the low GSI annual profiles (1 to 4%) during the months of October through March when any attempt at hormone-induced oocyte maturation and ovulation would be ineffective (Figure 2.3).

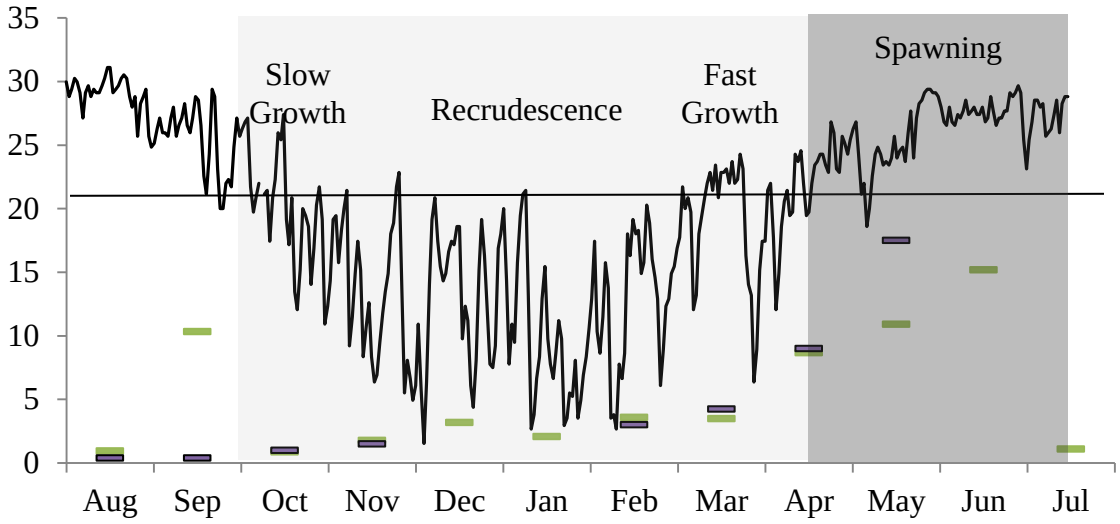


Figure 2.3 A composite profile of the monthly mean gonadosomatic index of channel catfish and the daily mean ambient air temperature (C) at the Aquaculture Research Station, Louisiana State University Agricultural Station, Baton Rouge, Louisiana. The mean GSI from each study (Trant et al. 1997, Banks et al. 1999) was denoted with different bar colors. The ambient air temperature data was obtained from the Southern Regional Climate Center, Louisiana State University, courtesy of ACIS (Applied Climate Information Systems) and CLIMOD (Climate Information for Management and Operational Decisions).

In an effort to obtain additional biological insight into channel catfish ovarian development, a new method of ovarian assessment using basic grey-scale ultrasound imaging was explored at

the Louisiana State University Agriculture Center – Aquaculture Research Station (LSUAC-ARS). Initial work showed that this imaging technology minimized fish handling and had strong potential utility in reproduction research and commercial-scale genetic improvement efforts. Thus, standard handling and imaging techniques to rapidly view the ovaries of unrestrained, submersed, unanesthetized channel catfish were developed and subsequently used throughout this dissertation (Guitreau et al. 2012).

Ultrasound imaging interconverts electrical and sound energy to produce and receive sonic frequencies (> 20,000 Hertz; usually 1 to 10 MHz) that are transmitted through a medium (such as water or ultrasound transmission gel) to produce images of internal anatomy (Toal 1996; Novelo and Tiersch 2012). The outstanding feature of this technology is that it can provide quick, non-invasive insight into internal biological processes that allows diagnosis of disease or reproductive processes without relying on invasive procedures such as surgery and biopsies, or highly specialized and time-consuming techniques such as histology or blood-hormone analysis that typically yield results days or weeks after sample collection.

The main challenge for using ultrasonography in channel catfish reproduction was that prior to this work there was no published systematic approach or methodology for interpretation of ultrasound images for assessment of channel catfish ovarian development. Training and learning of this procedure were needed to obtain biological insight on the complex biological processes of ovarian and oocyte development seen by use of this imaging technology (Figure 2.4).
The ultrasound images are produced by the presentation of dots (pixels) that are ultrasound waves electronically converted to represent different degrees of brightness on a grey-scale

(echogenicity) based on the amplitude and depth of the individual echo origin, which is modulated by the control settings used (real-time B-Mode). These control settings are essential to reproducibility and standardization of ultrasound imaging procedures in fish reproduction. The image below was generated using ultrasound controls set at 8 Mhz (frequency), 80 mm penetration depth, 17 mm focus depth, 56 dB dynamic range, 70% power, 80% Zoom, 100% View Area, 0 Rejection (this control has no unit of measure, numbers on slide bar control range from 0 to 12), with Time-Gain Compensation adjusted at 0, 20, 40, 60 and 80 mm (Figure 2.4 B).

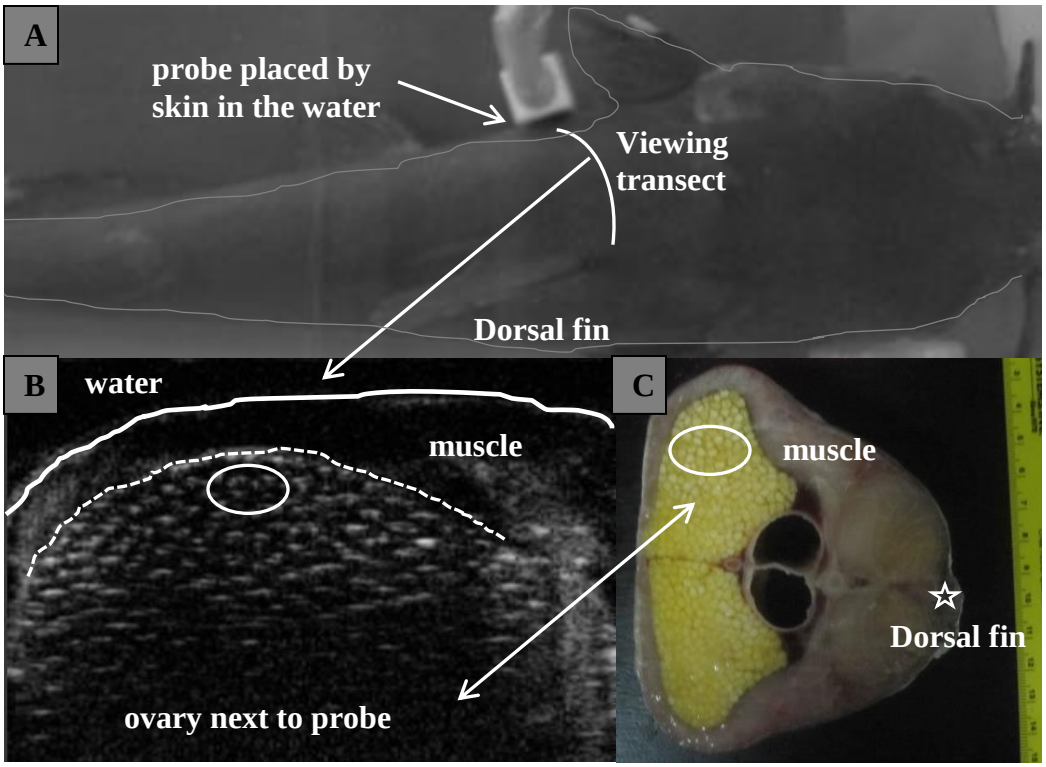


Figure 2.4 Ultrasound imaging with the probe placed by the left ovary (A), with the near-field view (40 mm depth) of the ultrasound image displayed (B) and the corresponding frozen cross-section of the channel catfish (C) produced images of the main tissue structures including the skin (curved line), muscles, the ovary and the oocytes (encased in the white oval).

Ultrasound imaging equipment can vary in cost from less than US$ 10,000 for basic grey-scale imaging (similar to the ultrasound unit used in this dissertation, which cost $10,000) to greater

than US$100,000 for units used in human medicine. Studies of channel catfish ovarian development in this dissertation were based on the use of a portable ultrasound imaging unit (Classic TelaVet 1000™ Veterinary Digital Ultrasound Module, Telemed UAB, Vilnius, Lithuania) and Real-Time B-Mode ultrasound controls. The control settings and ultrasound imaging procedures used were reported in each chapter because of their relevance to the standardization approach used in the development of interpretation and classification procedures for evaluation of channel catfish ovaries. This was done to provide guidance and to contribute towards development of a universal standardization platform for the use of this imaging technology in fish reproduction.

The use of ultrasonography in channel catfish reproduction would provide the benefit of immediate (< 30 sec) display of internal ovarian morphology relevant to assessing ovarian development. This unprecedented direct display of the reproductive state of the channel catfish ovary would help to decide whether or not to select fish for injection in genetic improvement efforts. This is especially relevant to commercial production of C × B hybrid during which ovarian assessment is currently only inferred by the use of external assessment methods. Ultrasound can help to identify females which would not produce viable eggs, and to identify females which would produce viable eggs, thus increasing the efficiency of production. This improved efficiency at a commercial-scale would reduce the overhead costs associated with labor, hormones, and time associated with the selection of females for artificial spawning. A review of the use of ultrasonography was presented first in an effort to provide the necessary background information for decision-making using ultrasonography.

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Chapter 3*
A Review of the Use of Ultrasonography in Fish Reproduction

Ultrasonography utilizes technology that interconverts electric and acoustic energy to create an image of internal anatomy, typically as gray-scale images produced for human and veterinary diagnostics. These images are created by different modes of ultrasound echo reception and display, including A-Mode (amplitude mode), B-Mode (brightness mode), Real-Time B-Mode (moving gray-scale images), M-Mode (motion, or time-motion mode), and color modes (introduced by Doppler technology). A-Mode produces one-dimensional displays of echo amplitudes, displayed as spikes on a vertical line. It is used especially for ophthalmic examination and for evaluating the fat and lean portions of meat in animals; it is least frequently used in reproduction studies (Ginther 1995). B-Mode produces two-dimensional gray-scale images, such that the brightness displayed by dots (or pixels) on the echo display screen corresponds to the amplitude of the individual echo signal, and the position of the dot corresponds to the depth of echo origin (Nyland et al. 2002).

Real-Time B-Mode creates a moving, cross-sectional gray-scale image. This, the most commonly used mode for examining the reproductive tract of animals, is composed of multiple B-Mode lines created by an ultrasound beam swept across a triangular (sector) or rectangular (linear) field of view. This yields a triangular- or rectangular-shaped ultrasound image displayed on the monitor (Nyland et al. 2002). The sector format may be produced by different probe types

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such as mechanical sector scanners, or by electronic steering of the ultrasound beam by a probe composed of multiple elements ("arrays"), including curvilinear arrays, curved arrays, and phased arrays (Nyland et al. 2002). The rectangular format is produced by the linear array probe which has multiple, rectangular, piezoelectric elements. The M-mode, an adaptation of the B-Mode, is used for evaluating moving structures such as the heart, and the motion of the dots, or the change in distance between the probe and the reflecting interfaces, is displayed on depth and time axes. The modes utilizing Doppler technology register the motion of blood toward, away, or at an angle to the transducer (probe) to construct color images of flow patterns. Ultrasound imaging technology refers to one or a combination of these modes of ultrasound echo reception and image display.

Ultrasonography, which provides direct, visual access to internal anatomy, has revolutionized access to biological data to monitor and diagnose changes in internal anatomy. This imaging technology was used in humans in the 1970s to study heart (Gowda et al. 2004), musculoskeletal structures (Kane et al. 2004), and the reproductive system (Jansen and van Os 1989). Ultrasonography also has been widely used for reproductive applications in farmed animals (Medan and Abd El-Aty 2010). In aquatic species, ultrasonography has been used to study reproduction in aquatic mammals, such as the bottlenose dolphin *Tursiops truncatus aduncas* ((Brook et al. 2000; Brook 2001; Robeck et al. 2005), the Indo-Pacific humpback dolphin *Sousa chinensis* (Brook et al. 2004), and killer whales *Orcinus orca* (Robeck et al. 2004); in amphibians, such as the tomato frog *Dyscophus antongili* (Schildger and Triet 2001); and in reptiles, such the American alligator *Alligator mississippiensis* (Lance et al. 2009).

In fish, ultrasonography has been used to study (1) internal structures such as muscle mass in channel catfish *Ictalurus punctatus* (Bosworth et al. 2001), thyroid gland in spotted dogfish *Scyliorhinus stellaris* and small-spotted catshark *S. canicula* (Gridelli et al. 2003), blood flow and vein contractions in the common cuttlefish *Sepia officinalis* (King et al. 2005) and (2) disease, such as liver tumors in zebrafish *Danio rerio* (Goessling et al. 2007), ocular lesions in halibut *Hippoglossus hippoglossus* (Williams et al. 2007), and internal disorders in ornamental common carp *Cyprinus carpio* (Saint-Erne 2010). Ultrasonography has also been used to study fish reproduction for nearly 30 years (Table 3.1).

The goal of this review was to assemble a comprehensive reference data set to serve as a decision-enabling tool for potential users. The objectives were to identify ultrasound equipment, settings, and procedures used during examination; review fish handling procedures used during examination; and review current data on sex identification and reproduction indices developed using ultrasonography. This review identified a large number of inconsistencies and omissions in reporting of equipment settings, fish handling, and description of the ultrasound imaging procedures that are relevant to reproduction studies using ultrasonography, whether in commercial or public hatcheries, or in field studies.

Our review summarizes contributions made by use of ultrasonography in fish reproduction and identifies approaches for improving throughput, fish handling, and reporting of equipment and instrument settings used for imaging. Application of ultrasonography in fish reproduction can be improved by collecting and routinely reporting the information necessary to replicate, standardize, and optimize imaging procedures.

Table 3.1 Studies of the use of ultrasonography in fish reproduction. The key listing includes the study identification number (ID), the common and scientific names (based on Nelson et al. [2004] whenever possible), the goal of the study, and references. Fish (N = 21 species) were grouped into two main categories—freshwater (24% of species) and marine and anadromous (76%)—according to family, genus, and species. The studies are listed chronologically for each species within a taxonomic order. The goal of each study is identified as sex identification (SI), the development or application of reproductive indices (RI), or both (SI, RI). Sex identification and reproductive indices were divided into two types of data: data based solely on ultrasonography (SI-1, RI-1) and those derived from both ultrasonography and other methods (SI-2, RI-2).

ID	Species	Goal	Reference
	Freshwater fishes		
1	Stellate sturgeon *Acipenser stellatus*	SI-1, RI-2	Moghim et al. (2002)
2	Shovelnose sturgeon *Scaphirhynchus platorynchus*	SI-1, RI-2	Colombo et al. (2004)
3	Shovelnose sturgeon	SI-2	Wildhaber et al. (2005)
	Pallid sturgeon *S. albus*	SI-2	
4	Shovelnose sturgeon	RI-2	Wildhaber et al. (2007)
5	Shovelnose sturgeon	RI-2	Bryan et al. (2007)
	Pallid sturgeon	RI-2	
6	Neosho madtom *Noturus placidus*	RI-2	Bryan et al. (2005)
7	Murray cod *Maccullochella peelii*	SI-1, RI-2	Newman et al. (2008)
	Marine and anadromous fishes		
8	Pacific herring *Clupea pallasii*	SI-1, RI-2	Bonar et al. (1989)
9	Atlantic cod *Gadus morhua*	SI-2	Karlsen and Holm (1994)
10	Atlantic cod	RI-1	Davie et al. (2003)
11	Atlantic cod	SI-2	McEvoy et al. (2009)
12	Barfin flounder *Verasper moseri*	SI-1	Matsubara et al. (1999)
13	Atlantic halibut *Hippoglossus hippoglossus*	RI-1	Shields et al. (1993)
14	Atlantic halibut	SI-1, RI-2	Martin-Robichaud and Rommens (2001)
	Winter flounder *Pseudopleuronectes americanus*	SI-1, RI-2	
	Yellowtail flounder *Limanda ferruginea*	SI-1, RI-2	
	Haddock *Melanogrammus aeglefinus*	SI-1, RI-2	
15	Haddock	RI-2	Martin-Robichaud and Berlinsky (2004)

Table 3.1. Continued.

ID	Species	Goal	Reference
16	Atlantic salmon *Salmo salar*	SI-1, RI-2	Mattson (1991)
17	Coho salmon *Oncorhynchus kisutch*	SI-1	Martin et al. (1983)
18	Steelhead[a] *O. mykiss*	SI-2, RI-2	Evans et al. (2004a)
19	Steelhead	RI-2	Evans et al. (2004b)
20	Striped bass *Morone saxatilis*	RI-2	Will et al. (2002)
21	Striped bass	SI-2, RI-2	Blythe et al. (1994a)
	Striped bass × white bass *M. chrysops*	SI-2	
22	Striped bass	SI-2, RI-2	Blythe et al. (1994b)
23	Striped bass	RI-2	Jennings et al. (2005)
24	Red hind *Ephinephelus guttatus*	SI-1, RI-2	Whiteman et al. (2005)
25	Nurse sharks *Ginglymostoma cirratum*	RI-2	Carrier et al. (2003)
26	Broadnose sevengill shark *Notorynchus cepedianus*	RI-1	Daly et al. (2007)
27	Small-spotted catshark *Scyliorhinus canicula*	RI-2	Whittamore et al. (2010)
	Thornback ray *Raja clavata*	RI-2	

[a]Anadromous form of rainbow trout.

Methods

Literature Collection. Search Strategy and Selection Criteria. Four databases (afsjournals.org, googlescholar.com, isiknowledge.com, onlinelibrary.wiley.com) were searched for peer-reviewed articles addressing ultrasound technology and reproduction in fish, representative of the literature in scientific journals published in English. The following key words used were in alternative combinations: "ultrasound," "fish," "reproduction," and "imaging." The "References" and the "Times Cited" links for each of the articles listed in the results of the main search query were searched. Based on this scheme, the search was extended into additional links and to the references of publications with titles explicitly stating the use of ultrasonography in fish reproduction. Eight percent (27 publications) of the 327 studies that were initially identified for review were selected based on their direct relevance to ultrasonography in fish reproduction. A key list was created for these studies, including a study identification number, with corresponding species names and references, for use in this review (Table 3.1).

Data Extraction. Publications were reviewed for the specifics reported on equipment and settings, biological data of fish, fish handling procedures, use of ultrasonography for sex identification, and development and application of reproductive indices. Data were compiled on (1) the ultrasound instrument, probe (transducer), storage device used, and corresponding technical procedures and settings; (2) biological data of fish, including the sample number, the number of male and female fish, age (years), length (cm), weight (g, kg), and reproduction terminology used for identifying life stage and reproductive condition; (3) fish handling procedures before and during ultrasonography; and (4) description of the scanning procedure. An EndNote Library and an Excel spreadsheet detailing the goal, common and scientific names, and

corresponding biological, fish handling, and ultrasound technique of each study were compiled. If data on ultrasound practices in fish reproduction were not reported, or explicitly stated, the data were classified as "NR" (not reported).

Results

Data Extracted. Equipment and Settings Used. The ultrasound unit and control settings – The model name of the ultrasound unit, along with the probe features reported, were compiled (Table 3.2). Most (96%) of the studies reported a model name for the ultrasound unit used, and 41% reported the use of portable machines suitable for use outside of the laboratory. However, the majority of the studies (89%) did not report the control settings used for obtaining ultrasound images. Use of the B-mode echo display was reported for four studies (Table 3.2). Although control settings such as focus depth, output power, and frame rate were reported by 11% of the studies (Table 3.2), the values for these settings were not reported, except for a single study that reported a specific value for "gain" and two studies that reported a specific value for "power" (Table 3.2).

The Probe: Features, Frequency Used and Procedures – The most reported probe features (Table 3.2) were frequency capability (96% of the studies) and the array (linear, sector, annular) formats (81% of the studies). The majority of the studies (74%) used a linear array, which produces rectangular-shaped images. Fewer studies (11%) used sector-type probes that produce triangular-shaped images, including a mechanical sector probe (study 14), a curvilinear array probe (study 26), and a comparison of the use of curved array and linear array probes (study 23). Most of the studies (96%) reported either a single ultrasound frequency or multiple frequencies as a probe attribute (Table 3.2).

Table 3.2 Compilation of data from publications for ultrasound imaging equipment (ultrasound unit and probe), the storage and formatting of images, and the corresponding technical procedures and settings used in fish reproduction studies. The following are reported for the ultrasound units: model, portability, echo display mode used (M), and basic control settings (CS) used for obtaining images (P = power settings [control of voltage, and thus the intensity of sound output by the probe], G = overall gain [causing uniform amplification of all returning echoes regardless of depth of origin], reject settings [causing suppression of returning echoes], and time-gain compensation settings [controlling near to far field amplification of returning echoes]). The probe features include the model, whether it was single or multiple frequency (S/M), the available frequencies (MHz), and the arrangement and shape (array) of the piezoelectric crystal elements, which produced linear (L), annular (A), sector (S), curved (C), and convex sector–linear (CL) image constructs. The data on probe use with regards to frequency transmission procedures and settings included whether the probe was covered, whether it was submersed, what frequency setting (FS) was utilized, and what medium of ultrasound transmission was used (ultrasound transmission gel or water). The ultrasound image storage device (SD) and whether formatting (F) of the ultrasound image was provided were reviewed. (Arr = array; Sub = submersed; Cov = Covered; FU = Frequency used (MHz); R = Reported; NR = not reported).

		Ultrasound unit[a]			Probe features				Probe use				Image	
ID	Species	Model	M	CS	Model	S/M	MHz	Arr[b]	Cov	Sub	FU	Medium	SD	F
1	Stellate Sturgeon	Pie Medical 200 VET	R	NR	R	NR	5/7.5	L	R	NR	5, 7.5	Water	R	NR
2	Shovelnose Sturgeon	Sonosite 180 Plus, portable	NR	NR	R	NR	5	L	NR	NR	5	Gel	NR	NR
3	Shovelnose and pallid Sturgeons	Shimadzu SDU-400 Plus;	NR	G	NR	NR	7.5	L	R	NR	7.5	Gel	NR	NR
		Sonosite 180 Plus, portable	NR	G	NR	M	5-10	L	R	NR	NR	Gel	NR	NR
4	Shovelnose Sturgeon	Shimadzu SDU-400 Plus;	NR	NR	NR	NR	7.5	L	R	NR	7.5	Gel	NR	NR
		Sonosite 180, portable	NR	NR	NR	M	5-10	L	R	NR	NR	Gel	NR	NR
5	Shovelnose and pallid Sturgeons	Shimadzu SDU-400 Plus[c]	NR	NR	NR	NR	7.5	L	NR	NR	7.5	NR	NR	NR
6	Neosho Madtom	GE LOGIQ 700 Expert[c]; Shimadzu	NR	NR	NR	S	8, 13	NR	NR	NR	8, 13	NR	NR	NR
		SDU-400 Plus[c]	NR	NR	NR	NR	7.5	NR	NR	NR	7.5	NR	NR	NR

Table 3.2 Continued.

		Ultrasound unit[a]			Probe features				Probe use				Image	
ID	Species	Model	M	CS	Model	S/M	MHz	Arr[b]	Cov	Sub	FU	Medium	SD	F
7	Murray cod	Sonosite 180 Plus;	NR	NR	NR	NR	5	L	NR	NR	5	NR	R	NR
8	Pacific Herring	Unirad EDP 1000 B scanner	NR	NR	NR	NR	5	NR	NR	R	5	NR	NR	NR
9	Atlantic cod	Pie Medical Scanner 450 VET	R	P	NR	NR	3.5	L	NR	R	3.5	Water	NR	NR
10	Atlantic cod	NR, portable;	NR	NR	NR	NR	7.5	NR	NR	NR	7.5	NR	NR	NR
11	Atlantic cod	Aloka SSD 500, portable[c]	NR	NR	NR	NR	7.5	L	NR	NR	7.5	NR	NR	R
12	Barfin Flounder	Echo Camera SSD-1000[c]	NR	NR	R	NR	10	A	NR	NR	10	No gel	NR	NR
13	Atlantic Halibut	Aloka model 210DX11[c]	NR	NR	NR	NR	7.5	L	NR	R	7.5	Mucus	R	VR
14	Atlantic Halibut	Ultramark 4 Plus[c]	NR	NR	R	M	5, 7.5, 10	S	NR	R	5, 7.5	NR	R	VR
	Winter and Yellowtail Flounder	Ultramark 4 Plus[c]	NR	NR	NR	M	5, 7.5, 10	S	NR	R	5	NR	R	VR
	Haddock	Ultramark 4 Plus[c]	NR	NR	NR	M	5, 7.5,	S	NR	R	NR	NR	R	VR
15	Haddock	Ultramark 4 Plus[c]	NR	NR	NR	NR	NR	NR	NR	NR	5	NR	R	VR
16	Atlantic Salmon	Pie Medical Scanner 450 VET	NR	P	NR	NR	3.5	L	NR	NR	3.5	NR	NR	NR
17	Juvenile coho salmon	Xenotec XUC-4[c]	NR	NR	NR	NR	15	NR	NR	R	15	Gel	NR	NR
	Adult coho Salmon	Advance Technology Laboratories Mark V[c]	NR	NR	NR	NR	5	NR	NR	NR	5	Gel	NR	NR
18	Steelhead[d]	Aloka SSD-500v, portable	NR	NR	NR	NR	7.5	L	NR	NR	7.5	NR	NR	NR
19	Steelhead	Aloka SSD-500v, portable	NR	NR	NR	NR	7.5	L	NR	NR	7.5	NR	R	NR

Table 3.2 Continued.

		Ultrasound unit[a]			Probe features				Probe use				Image	
ID	Species	Model	M	CS	Model	S/M	MHz	Arr[b]	Cov	Sub	FU	Medium	SD	F
20	Striped bass	Pie Medical Scanner 100LC, portable	NR	NR	NR	M	6/8	L	NR	NR	NR	NR	R	VR
21	Adult striped bass, hybrids[e]	Aloka 500 V	R	NR	NR	NR	5	L	NR	NR	5	NR	NR	NR
22	Striped bass	Aloka 500Z	R	NR	NR	NR	5	L	NR	NR	5	NR	R	VR
23	Striped bass	Pie Medical Scanner;	NR	NR	NR	M	3.5/5.0	C	NR	NR	NR	NR	R	NR
		LC100, portable			NR	M	6.0/8.0	L	NR	NR	NR	NR	R	NR
24	Red hind	Pie Medical Scanner, portable	NR	NR	NR	M	3.5–5.0	L	NR	NR	NR	NR	R	NR
25	Nurse Sharks	Pie Medical Scanner, model 200[c]	NR	NR	R	NR	3.5	L	NR	NR	3.5	NR	R	NR
26	Broadnose Sevengill Sharks	Aloka SSD500[c]	NR	NR	NR	NR	3.5	CL	R	R	3.5	Gel	R	NR
27	Small-Spotted Catshark	Concept MCV, portable[c]	NR	NR	NR	NR	7.5	L	No	R	7.5	SW[f]	R	NR
	Thornback Ray	Concept MCV, portable[c]	NR	NR	NR	NR	7.5	L	No	R	7.5	SW[f]	R	NR

[a]Names as reported in publications
[b]L = linear, S = mechanical sector, C&L = convex sector–linear scanner, A = annular, C = curved
[c]Manufacturer and address were reported for the model of ultrasound machine or probe used
[d]Anadromous rainbow trout
[e]Juvenile and adult striped bass × white bass
[f]SeaWater.

Five of seven studies that reported multiple frequency values of the probe did not specify the actual frequency used during ultrasound imaging (Table 3.2).

Ultrasound frequencies used in all the studies reviewed ranged from 3.5 to 15 MHz. The frequencies used for acipenserid fish of 44–150 cm fork length (FL) were 5 and 7.5 MHz; those for salmonid fish of 10–45 cm FL were 3.5, 5, 7.5, and 15 MHz; those for moronid fish of 55–100 cm total length (TL) were 3.5, 5, 6, and 8 MHz; and those for pleuronectid fish were 10 MHz for fish 1–40 TL and 5 and 7.5 MHz for fish 54–120 cm FL. The procedure reported for use of the ultrasound probe and for ultrasound frequency transmission included covering the probe (15% of studies), not covering the probe (4%), completely submerging the probe in water (26%), using water as an ultrasound transmission medium (11%), and using gel as an ultrasound transmission medium (22%).

The Storage Device and Ultrasound Image Format – The type of storage device used for recording images was reported by 48% of the studies reviewed (Table 3.2). The main storage device was videotape. One study identifying the use of a videotape recorder reported photographing recorded images displayed on the monitor; another reported the use of thermal print images, digitized into Tagged Image Format Files (TIFF). Studies that reported video recording of ultrasound images generally did not report the image format.

Handling Procedures. Handling before Ultrasonography – The holding systems (tanks mostly) were used as containers during ultrasonography in 13 studies for steelhead, striped bass, broadnose sevengill sharks, Atlantic halibut, and haddock (Table 3.3).

Table 3.3 Compilation of data on fish handling procedures, including the holding system (HS) such as tanks, raceways, or ponds in the laboratory or field, the container used to hold fish during ultrasonography, whether or not the fish was killed before imaging, and whether or not the fish was submersed (Sub) in water, physically restrained (Res), or anesthetized (Anes). Data on fish position (Pos), the external anatomy scanned (scan region), and the duration of the ultrasound imaging procedure (time/fish) are also listed. Yes = reported in the study; NR = not reported in the study.

ID	Species	HS	Container	Killed	Sub	Res	Anes	Pos[a]	Scan region	Time/fish
1	Stellate sturgeon	NR	Tank	No	Yes	NR	Yes	NR	1 cm from the skin, on the lateral sides, between the pectoral and anal fins	<30 s
2	Shovelnose sturgeon	NR	NR	Yes	NR	Yes	Yes	NR	On the left side, above the third and fourth ventral scutes anterior to the pelvic fins	NR
3	Shovelnose sturgeon	Flow-through circular tanks	NR	No	Yes	NR	No	D	Ventral abdominal surface from vent to opercula	NR
	Pallid sturgeon	NR	NR	No	Yes	NR	No	D	Ventral abdominal surface from vent to opercula	NR
4	Shovelnose sturgeon	Flow-through circular tanks	NR	No	Yes	NR	No	D	Ventral abdominal surface from vent to opercula (illustrated) and alongside, ventrally, between the row of belly scutes and the first row of side scutes	NR

Table 3.3 Continued.

ID	Species	HS	Container	Killed	Sub	Res	Anes	Pos[a]	Scan region	Time/fish
5	Shovelnose sturgeon	NR	NR	No	NR	NR	NR	NR	Three equidistant points along the gonad	NR
	Pallid sturgeon	NR	NR	No	NR	NR	NR	NR	Three equidistant points along the gonad	NR
6	Neosho madtom	720-L living stream	A pan of water	No	NR	NR	Yes	D	Ventrally, against the abdomen	NR
7	Murray cod	2500-L circular tanks	100-L bath	Yes	Yes[b]	Yes	Yes	D	Midlateral region, ventral	<1 min
8	Pacific herring	On ice	10-cm-deep water bath	Yes	Yes	Yes	No	L	Anterior to the dorsal fin, and perpendicular to the long axis of the fish	"Within seconds"
9	Atlantic cod	NR	Glass aquarium	No	Yes	NR	Yes	D	Ventral side up, caudal part of abdominal cavity (illustrated)	<30 s
10	Atlantic cod	6.5-m3 tanks	NR	No	NR	NR	NR	NR	NR	NR
11	Atlantic cod	In large tanks	Yes[c]	No	NR	NR	Yes	NR	Coelomic cavity	20–40 s
12	Barfin flounder	In aquarium	Shallow plastic tray	No, Yes	NR	Yes	No	L	The skin on abdomen, moving laterally (illustrated)	2 min
13	Atlantic halibut	4.5-m-diameter tanks	Holding system	No	Yes	No	No	L	The skin above the ovary, on three positions, and the entire ovary (illustrated)	NR

Table 3.3 Continued.

ID	Species	HS	Container	Killed	Sub	Res	Anes	Pos[a]	Scan region	Time/fish
14	Atlantic halibut	NR	44-L container	No	Yes	NR	Yes	L	On side of fish, flatfish (lying flat), directly posterior to the gut region (illustrated)	NR
	Winter flounder	NR	44-L container	No	Yes	NR	Yes	L	On side of fish, flatfish (lying flat), directly posterior to the gut region(illustrated)	NR
	Yellowtail flounder	NR	44-L container	No	Yes	NR	Yes	L	On side of fish, flatfish (lying flat), directly posterior to the gut region (illustrated)	NR
	Haddock	NR	76-L container	No	Yes	NR	Yes	D	Ventral side up, directly anterior to the urogenital pore (illustrated)	NR
15	Haddock	147-m^3 tanks	Holding system	No	Yes	NR	Yes	NR	NR	NR
16	Atlantic salmon	NR	Tank	No	Yes	NR	Yes	D	Upside down, over the belly, back and forth over the belly	NR
17	Coho salmon	NR	On a rotating platform	No, Yes	Yes	Yes	Yes	NR	At various posterior distances from the gill and ventral distances from the lateral line	NR
18	Steelhead[d]	Temporary 190-L tank	Holding system	No	Yes	NR	Yes	D	Moved anterior or posterior on ventral side of abdomen	NR

Table 3.3 Continued.

ID	Species	HS	Container	Killed	Sub	Res	Anes	Pos[a]	Scan region	Time/fish
19	Steelhead	Temporary 190-L tank	Holding system	No, Yes	NR	Yes	Yes	NR	Along abdominal surface	4 min
20	Striped bass	NR	NR	No	NR	Yes	NR	NR	Oriented probe perpendicular or parallel to fish body until ovary visible	NR
21	Striped bass	2200-L tanks; raceways	180-L tank	No	Yes	NR	Yes	V	Midlaterally along length of gonad, at base of pelvic fin, at isthmus of dorsal fin	<1 min
	Hybrids[e]	NR	NR	NR	NR	NR	NR	NR	NR	NR
22	Striped bass	2,200-L tanks; raceways	Tank	No	Yes	NR	Yes	V	Midlaterally along length of gonad, at base of pelvic fin at isthmus of dorsal fin	NR
23	Striped bass	Holding tanks	Holding tanks	No	NR	NR	No	D	Against the ventral side (abdomen) of the fish (illustrated)	3–4 min
24	Red hind	NR	NR	No, Yes	NR	Yes	NR	NR	Along ventral surface, at four intervals from the vent to the anterior section of the ovary	NR
25	Nurse sharks	Temporary enclosures; pools[f]	1 × 1 × 3 m transport unit	No	Yes	Yes	Yes	D	NR	NR

Table 3.3 Continued.

ID	Species	HS	Container	Killed	Sub	Res	Anes	Pos[a]	Scan region	Time/fish
26	Broadnose sevengill sharks	Oceanarium tanks	Holding system	No	Yes	Yes	No	D	Anterior to the pectoral girdle and finishing at the pelvic girdle (illustrated)	≤10 min
27	Small-spotted catshark	1090 × 610 × 1,020 mm tanks	1,230 × 550× 260 mm tanks	Yes	Yes	Yes	No	Tilted	Ventral surface, along the abdomen, on ventral left side (illustrated)	NR
	Thornback ray	1091 × 610 × 1,020 mm tanks	1,231 × 550× 260 mm tanks	Yes	Yes	Yes	No	V	1–3 cm above dorsal surface, in a lateral orientation (illustrated)	NR

[a]V = ventral recumbency (held upright, in swimming position; does not include flatfishes, which were in swimming position but scanned on one side [lateral recumbency]); D = dorsal recumbency (held ventral side up, upside down, inverted, supine position); L = lateral recumbency (left to lie on one side, fish placed on side).
[b]Partially submersed.
[c]In this study fish were transferred to bucket for anesthesia, but whether the bucket was the container used during ultrasonography was not specified.
[d]Anadromous form of rainbow trout.
[e]Juvenile and adult striped bass × white bass.
[f]Temporary enclosures; pools: temporary enclosures in field; indoor (12 m diameter, 1.5 m deep) and outdoor (10 m length, 4.5 m width, 1.5 m deep) pools.

Containers other than the holding systems were used for the Neosho madtom, Murray cod, barfin flounder, striped bass, nurse sharks, small spotted catshark, and the thornback ray. The majority (70%) of studies reported use of live fish during ultrasound imaging;

a few studies (22%) reported killing fish as a standard procedure before imaging; two studies reported killing fish for ease of transportation (study 12) or as a consequence of handling (i.e., air embolism; study 24).

Handling Leading up to Scanning. In 63% of the studies fish were completely submersed, including fish that were alive (e.g., broadnose sevengill shark) or dead (e.g., Pacific herring); in one study, fish (Murray cod) were partially submerged. Several (37%) of the studies did not report whether the fish remained submersed in water (Table 3.3).

The majority (78%) of the studies involved procedures that physically (10 studies) or chemically (15 studies) restrained the fish. Fish categorized as physically restrained included fish that were killed as part of the sampling procedure (shovelnose sturgeon, Murray cod, Pacific herring, barfin flounder, steelhead, small-spotted catshark, and thornback ray), those unintentionally killed as a result of handling (red hind), those sampled during electrofishing (striped bass), and those physically enveloped in a vinyl bag (broadnose sevengill sharks). The fish that were chemically restrained (use of anesthesia) included stellate sturgeon, Neosho madtom, Atlantic cod, Atlantic salmon, Atlantic halibut, winter and yellowtail flounders, haddock, steelhead, striped bass, and nurse sharks (Table 3.3). One study physically and chemically restrained juvenile coho salmon, which were anesthetized and strapped onto a rotating platform. One of the 27 studies (adult Atlantic halibut, 12–25 kg) did not use any chemical or physical restraint, and a few studies (11%) did not use anesthesia because fish (shovelnose sturgeons 65–74 cm TL, 1 kg; striped bass 60–100 TL, 3–19 kg) were docile after being positioned ventral side up.

Handling during Scanning – The majority of the studies (63%) reported the physical positioning of the fish (Table 3.3). Fish held in dorsal recumbency (in an inverted position) included shovelnose sturgeon, pallid sturgeon, Neosho madtom, Murray cod, haddock, Atlantic salmon, steelhead, striped bass, nurse sharks, and broadnose sevengill sharks. Fish held in ventral recumbency (upright swimming position) included striped bass and thornback ray. Fish held in lateral recumbency (on their side) were mostly flatfishes, except for Pacific herring.

As to the scanning region, 8 studies described the physical position of the probe on the fish by referencing external anatomy (in-text descriptions of the scanned region) and by providing a schematic representation (Table 3.3). The majority of the studies (59%) described the scanned region of the fish without illustration, or with descriptions ranging from specific anatomical references such as "midlaterally along length of the gonad, at the base of the pelvic fin, at isthmus of dorsal fin," with the fish in ventral recumbency, to general references such as the probe being perpendicular and parallel to the fish body.

For the duration (s, min) of ultrasound examination, 37% of the studies reported an estimate, such as "within seconds," a range (e.g., 3–4 min), or an estimated average time (ranging from <30 s to 10 min) of the ultrasound procedure per fish.

Biological Data. Overview of Fish Studied – The three taxonomic sub-classes of fish (Chondrostei, Neopterygii, and Elasmobranchii) were studied using ultrasonography for sex identification or development and for application of reproductive indices. The most studied groupings were marine and anadromous fishes, and the most studied species were shovelnose

sturgeon, striped bass, and Atlantic cod (Table 3.1). The goals of these studies (n = 27) were to use ultrasonography for sex identification (19%), measurement of reproductive indices (44%), or sex identification and measurement of reproductive indices (37%). Sex identification was accomplished mostly by the sole use of ultrasonography, followed by dissection and gross visual examination of gonads; ultrasonography was also used in combination with other methods such as endoscopy and histology and with mathematical modeling such as fractional analysis. Reproductive indices were developed on the basis of the sole use of ultrasonography, but also by integrating data from ultrasonography with results of other methods such as measurements of gross anatomy or the use of histology-based gonadal development indices.

In total, over 6,434 fish were used in all the studies combined (Table 3.4). Sixty-three percent (4,078 fish) of the total number was reported as being either male (1,195) or female (2,883). No study reported exclusively on male fish, whereas a third of the studies (30%) were exclusively on females, including haddock, striped bass, and elasmobranch fishes; the majority of the studies(56%) reported imaging of male and female fish. Length differences among species ranged from 10 to 14 cm TL for Neosho madtom to 265 cm TL for broadnose sevengill shark.

Life stage–dependent length differences ranged from 54 to 71 cm FL for juvenile Atlantic halibut, 10–25 cm FL for juvenile coho salmon, 35–45 cm FL for adult coho salmon, and 96–120 cm FL for adult Atlantic halibut (Table 3.4). Species-dependent weight varied between 68 and 94 g for Pacific herring and between 4 and 16 kg for adult striped bass (Table 3.4).

Table 3.4 Compilation of biological data on fish species, including the number sampled (No.), the numbers of males (M) and females (F), age (to the nearest year), length (fork [FL], standard [SL], total [TL], or body length to the nearest centimeter), weight (to the nearest kilogram unless specified otherwise), and the terminology used to describe life stage or reproductive condition. These values provided life stage and gonadal maturity data, which were linked to the results of ultrasonography for sex identification and the development of reproductive indices. In general, size was linked to ease of sex identification, and reproductive indices were developed for adult fish during the spawning season or at different stages of gonadal development during the spawning cycle; NR = not reported in the study.

ID	Species	No.	M	F	Age	Length	Weight	Terminology
1	Stellate sturgeon	249	50	199	6–16	95–150 FL	5–16	Mature, immature
2	Shovelnose sturgeon	51	25	25	NR	44–71 FL	NR	Spawning migration
3	Shovelnose sturgeon	343	183	160	16–19	65–74 TL	1	Adult
	Pallid sturgeon	16	11	3	NR	NR	NR	Adult
4	Shovelnose sturgeon	NR	NR	NR	NR	NR	NR	Adult
5	Shovelnose sturgeon	228	NR	NR	NR	>55 TL	NR	NR
	Pallid sturgeon	16	11	4	NR	NR	12–25	NR
6	Neosho madtom	58	22	36	1–3	10–14 TL	NR	Cyclic spawning condition
7	Murray cod	289	66	223	1–3	NR	1–4	Pubertal transition
		90	25	65	6	NR	6	Reproductively mature adults
8	Pacific herring	176	57	55	NR	16–23 SL	68–94 g	Mature, immature
9	Atlantic cod	788	NR	NR	1–6	NR	1–5	Maturing, nonmaturing
10	Atlantic cod	1,200	NR	NR	1–3	NR	1–3	Immature, mature
11	Baltic cod	32	16	16	NR	44–50 body length	1	Sexually mature
12	Barfin flounder	98	55	43	1–2	1–40 TL	NR	Immature
13	Atlantic halibut	NR	0	NR	NR	NR	12–25	Mature broodstock

Table 3.4. Continued.

ID	Species	No.	M	F	Age	Length	Weight	Terminology
14	Atlantic halibut	21	NR	NR	4	54–71 FL	2–5	Juvenile
	Atlantic halibut	NR	NR	NR	NR	96–120 FL	13–28	Mature
	Winter flounder	10	NR	NR	NR	NR	NR	Mature
	Yellowtail flounder	10	NR	NR	NR	NR	NR	Mature
	Haddock	25	0	25	NR	56–73 FL	NR	Mature
15	Haddock	58	0	58	NR	60–65 FL	2	Broodstock
16	Atlantic salmon	79	30	49	NR	NR	3–6	Two sea winter
17	Coho salmon	15	NR	NR	NR	10–25 FL	NR	Juvenile
	Coho salmon	5	NR	NR	NR	35–45 FL	NR	Mature
18	Steelheada	1,353	330	1,023	NR	NR	NR	Adult
19	Steelhead[a]	108+	58+	50+	NR	NR	NR	Adult
20	Striped bass	31	0	31	NR	58–98 TL	4–16	Adult
21	Striped bass	16	8	8	5	55 TL	3	Adult
	Hybrid striped bass[b]	46	12	27	1+	NR	NR	Juveniles
	Hybrid striped bass[b]	20	7	4	2+	NR	≤2	Adult
22	Striped bass	40	20	20	5	55 TL	3	Adult
23	Striped bass	28	0	28	NR	60–100 TL	3–19	NR
24	Red hind	25	NR	NR	NR	28–42 TL	NR	NR
		820	209	611	NR	28–42 TL	NR	Spawning aggregation
25	Nurse sharks	5	0	5	NR	NR	NR	Reproductively active
26	Broadnose sevengill shark	4	0	4	NR	240–265 TL	NR	Sexually mature
27	Small-spotted catshark	77	0	77	NR	38–70 TL	≤1	NR
	Thornback ray	34	0	34	NR	49–85 TL	1–4	NR

[a]Anadromous form of rainbow trout.
[b]Striped bass × white bass.

Terminology used for describing the reproductive condition of fish included life stage terminology, such as “juvenile,” and “adult”; gonadal maturity terminology, such as “immature,” and “mature”; and descriptions of reproductive activity of fish, such as “spawning migration” for shovelnose sturgeon and “spawning aggregation” for red hind (Table 3.4).

Sex Identification – The ease of sex identification generally increased with an increase in the size of the gonad, which corresponded to the adult life stage, and gonadal maturation of reproductively active fish during their spawning period. For instance, accuracy of sex identification for female stellate sturgeons was 99–100% for fish designated as mature and immature, 96% for male stellate sturgeons designated as mature, but only 76% for male stellate sturgeons designated as immature (Table 3.2). Overall accuracy of sex identification was 86% for shovelnose sturgeons designated as mature and immature (Table 3.2), 70% for shovelnose sturgeon, 86% for pallid sturgeon designated as adults, 78% for Baltic cod broodstock of more than 40 cm body length, and 95% for adult striped bass throughout their annual reproductive cycle. Accuracy of sex identification exceeded 90% for Atlantic cod when the gonads were largest, leading to spawning. However, one study on female Atlantic salmon reported that imaging the ovary became increasingly difficult as gonadal maturation advanced.

Ease of differentiating between male and female fish decreased for smaller-sized gonads. This was attributed to early life stages (juveniles, or young adults), to gonadal development during the reproductive cycle (previtellogenic, late vitellogenic, or atretic gonads), or to the morphology of the gonad regardless of life stage or gonadal maturity during a reproductive cycle. For example, the effect of juvenile life stages and the corresponding small size of the gonads on the inability to

identify gonads were reported for hybrid bass (striped bass *M. saxatilis* × white bass *M. chrysops*, including age-2 adult males), barfin flounder, and coho salmon (Table 3.2). Gonads of Atlantic cod were not visible until their gonad growth was distinct (study 9), and the gonads of red hind were reliably identified at the final stages of maturation only in the days immediately before spawning (study 24). Gonads were not identified for "immature" or "spent" female shovelnose sturgeon (study 3), "recovering" male and female Atlantic cod (study 9), and "atrophied" testes of steelhead (study 18). The "recrudescent" ovaries of shovelnose sturgeon were difficult to distinguish from testes (study 2). In some cases, the gonad could not be identified because of the morphology, regardless of life stage (e.g., juvenile, young adult), or the maturity status of reproductively active adults. In the Neosho madtom, for example, the testes of adult males could not be identified from surrounding organs in the ultrasound image (study 6).

Reproductive indices based on ultrasonography alone – Qualitative descriptions of gonadal growth, ovulation, and oocyte maturation were reported for Atlantic halibut monitored using ultrasonography only (study 13). Quantitative reproductive indices based on the sole use of ultrasonography were developed for Neosho madtom (study 5), Atlantic cod (study 10), steelhead (study 18), and broadnose sevengill sharks (study 26). For Neosho madtom, fecundity was calculated from ultrasound images of the ovary. For steelhead, the presence or absence of oocytes in ultrasound images was used to distinguish between prespawn and postspawn females, and a threshold area of 1.25 cm2 (cross-sectional testis area) was used for separating prespawn from postspawn males (study 18). For broadnose sevengill shark, ultrasonography was used to measure changes in follicle diameter over a period of 1–13 months and to develop a behavior index (study 26).

Reproductive indices based on ultrasonography combined with other methods – Descriptions of ultrasound images for different gonadal development stages were developed based on interpretation of structures in the ultrasound image and data derived from other gonadal assessment methods. For example, stellate sturgeon were designated as mature and immature based on previously developed classification stages (Lagler 1978); subsequently, ultrasound images for these mature and immature gonads were reported. For shovelnose sturgeon, data from endoscopic imaging, gross morphology, blood, and gonadal tissue sampling, along with qualitative descriptions of ultrasound images for six gonadal stages, were organized within the framework of histological profiles on gonadal development previously developed by Moos (1978).

The quantitative reproductive indices for shovelnose sturgeon (study 5), striped bass (studies 20, 23), and red hind (study 24) were based on gonad length obtained by using ultrasonography for identifying the anterior margin of gonads and then using a ruler to measure the length of the gonad to the posterior margin (for shovelnose sturgeon) or to the vent (for striped bass and red hind), on the external surface of the fish. These data were combined with the mean cross-sectional gonad area calculated from ultrasound images for estimating ovary volume. Based on these imaging measurements, a regression model was developed for predicting ovary volume for striped bass (study 23); fecundity estimates were calculated by integrating ovary volumes with data on oocyte diameter (from ultrasound images) for shovelnose sturgeon, egg enumeration (catheter sampling) for striped bass, and oocyte densities (catheter sampling) for red hind. For striped bass, a threshold ovary size (>30 mm diameter) was developed for characterizing “ripe” females by correlating maximum monthly ovary diameter obtained from

ultrasound images to egg diameter obtained from measuring sampled oocytes (catheter) with an ocular micrometer and dissecting microscope; threshold testes size (>20 mm diameter) was determined for spermiating striped bass (study 21). Threshold testes size (1.25 cm^2) was developed for separating prespawn from postspawn steelhead by corroborating ultrasound diagnosis with blood plasma steroid levels (Study 19). Other reproductive indices developed included the following: for shovelnose sturgeon, a volumetric analog of the Gonadosomatic Index [(gonad volume/total body volume) × 100, study 5]; for Murray cod, the Gonad Index [(the cross-sectional gonadal diameter/square root of the body weight) × 100, study 7]; for haddock, the gonadal index [(the area of one ovarian lobe/fork length) × 100, study 14] and the ovarian index [(ovarian area/fork length) × 100, study 15].

Discussion

Equipment and Settings Used. The Ultrasound Unit – There was great diversity in the size and portability of ultrasound units and probes used in the studies reviewed (Table 3.2). The prices of those units ranged from US$2,000–9,000 for used or refurbished units (e.g., http://www.sonomahealth.com/sonosite-180plus.html), but specialized machines can cost more than US$100, 000 for use in human medicine. Most studies (96%) reported the model of the ultrasound unit used, but reporting the model of the ultrasound equipment used was inconsistent. For instance, manufacturer information was reported by 44% of the studies; 52% reported only the ultrasound unit model and no manufacturer information; and one study reported the manufacturer information but did not report the model of the ultrasound unit used (Table 3.2). Furthermore, the probe model was rarely reported, even though the probe determines specific ultrasound features that cannot be changed during the ultrasound procedure (Nyland et al. 2002). For example, the probe may be of a single frequency, which necessitates physically replacing the

probe to use another frequency with the ultrasound unit, or a probe may have multiple frequencies, which are adjusted by user interface controls (e.g., software). The probe frequency, in turn, determines the wavelength, the pulse length (axial resolution), the elevation resolution, and the arrangement (array) of the piezoelectric crystals (Nyland et al. 2002). Higher frequencies yield higher resolution, shorter wavelengths, shorter pulses, narrower beam diameters, and less depth penetration into the tissue. Lower frequencies yield lower resolution, longer wavelengths, longer pulses, wider beam diameters, and deeper penetration into the tissue (Ginther 1995). Accordingly, there is a trade-off between imaging depth and image resolution.

Ultrasound Control Settings – The features available in the ultrasound unit enable users to select the mode of echo return (e.g., A-mode, B-mode, real-time B-mode). Although the mode of echo display was not reported in most studies, the use of real time B-mode reported by four studies indicated that this gray-scale imaging method (without use of color modes introduced by Doppler technology) was sufficient for assessing reproductive indices or for sex identification in the stellate sturgeon, Atlantic cod, and striped bass, and that it may be adequate for viewing anatomy for sex identification and development of reproductive indices in fish.

Surprisingly few studies (11%) reported the ultrasound control settings, and of these all the studies reported only a single control setting, for instance, only the power or gain used (Table 3.2). Ultrasound units have a variety of control knobs and sliders that can be manually or electronically adjusted, and the units are constructed in a variety of shapes and user interfaces, making available a suite of adjustments for imaging that are specific to a manufacturer's ultrasound models. Because of this variation in the way the settings are controlled, reporting

these settings will include important information. If the manufacturer, model information, and control settings are included in a study, other users of this technology will be able to replicate the imaging reported, particularly novice users who may consider purchasing the same model previously reported for a particular species. At a minimum, three basic types of imaging controls should be reported to ensure the process of replication, evaluation, standardization, and optimization of ultrasonography for reproduction studies among different groups of fish: power (intensity/output), gain and reject, and time-gain compensation (TGC) (Nyland et al. 2002) 2002). The power control regulates the voltage applied to the piezoelectric crystals within the probe, thus affecting the sound output; the gain and reject controls affect amplification of returning echoes; the TGC controls adjust or compensate for weaker echoes originating from near (near gain), intermediate (slope delay control), or far (far gain) depths of echo origin (Nyland et al. 2002). These settings, at a minimum, must all be reported for a work to be replicated.

The Probe: Features, Frequency and Procedures – If the model is identified as a single-frequency probe, most probably that frequency was the one used for imaging. However, if multiple-frequency probes are used, reporting of the actual frequency setting used during ultrasonography is necessary. If the frequency is specified, especially with multiple-frequency probes, users will be able to verify, replicate, or standardize technical procedures for generating images for specific fish or groups of fish. The procedures for using the probe are also relevant because they contain additional steps (e.g., use of gel as an ultrasound transmission medium, or covering the probe) necessary for repetition of studies.

Storage Device and Image Format – The types of storage equipment used for ultrasound image were videotapes, thermal print images, digital camera pictures, and the internal storage capability provided by the user interface of the ultrasound unit. The equipment, and the image formats, which are for the most part dictated by the manufacturer, indicate the procedure of the physical transfer of information and provide information on the potential limitation or utility of the type of format and quality obtained for further image processing and analysis.

Fish Handling. Fish handling is important to the experiment being conducted, and potential effects introduced by handling should be minimized or standardized, to eliminate or account for potential interactions with the variables being studied. In using ultrasonography in fish reproduction, proper handling is critical to reproductive processes, and if ultrasonography is to be justified in its utility as a noninvasive technique, so too should be the associated fish handling techniques. The conditions of the holding system, such as available oxygen and temperature, can accelerate or inhibit reproduction and determine whether the fish being held are dead (as a consequence of no oxygen, for instance) or alive. Equally important is the method of transfer of fish from the holding system to another container, which can be done in different ways depending on fish morphology, or on the techniques of different fish handlers. For instance, the size of the broadnose sevengill shark necessitates leaving the shark within the water and enclosing it with a trap for imaging. For catfishes, physical damage can be avoided by moving the fish quickly in dip nets between the holding system and the container for imaging (Novelo et al. 2011), rather than holding the caudal peduncle with one hand and the base of the head with the other hand. The potential harm of dropping broodstock fish, which can lead to mortality or disruption of reproduction, can be avoided by transferring with a net, and using noninvasive

procedures such as ultrasound imaging. Proper reporting of handling conditions and procedures will enable optimization and standardization of techniques that may be adopted by other researchers for similar species.

All of the studies indicated whether the fish were dead or alive during the imaging procedure, but an overview of fish handling showed considerable variability in the procedures used and in the manner in which the procedures were reported. For instance, 13 studies included data on both the fish-holding system in laboratory conditions and the container used in laboratory and field conditions; the rest of the studies reported only the holding system or the container used (Table 3.3). The data reported varied across the studies for whether or not fish were submersed, physically restrained, or anesthetized; description of the scanned region; and time of ultrasonography procedure per fish (Table 3.3). For anesthesia procedures, for example, 30% of studies included data on the anesthetic used (chemical name), and the dose; 15% of studies reported only the anesthetic used (chemical name) without dose, and 11% of studies that used anesthesia included data on neither the anesthetic used nor the dose. Studies describing the position of the fish during ultrasound scanning with the probe may or may not have included specific referencing of external anatomy as landmarks for the area scanned, or presented a figure (photograph, or schematic representation) illustrating the physical positioning of the probe and fish. Finally, although 10 studies reported an approximation of the time it took to obtain an ultrasound image per fish, this was the least reported data relevant to fish handling.

Recommendations. Reporting ultrasound equipment and settings – To move towards standardization of procedures and provide access to information for users (including future

potential adopters) of ultrasonography in fish reproduction, consistent reporting is necessary. The most important factors are identifying the equipment and settings used for obtaining ultrasound images. The manufacturer's model name and the manufacturer's contact information (company name and physical address, including internet address if available) should be reported for the ultrasound unit and probe used. Further, although the names used to identify control settings vary among ultrasound units, effort must be made to report the suite of settings that would enable other users of this technology to replicate, test, or optimize technical settings for particular species and life stages (e.g., for juveniles or adults). An additional advantage of manufacturers' preset options in some ultrasound units are that the control settings used for obtaining a particular image are saved in the memory of the unit and can be retrieved for obtaining images of biologically similar animals. Reporting of the ultrasound control settings and values used for generating images will enable users to replicate or experiment with settings reported in the literature, thereby making data collected with this technology available for use by others. This is critical for standardizing procedures among similar species, or within species for juveniles and for adults.

Reporting and Improving of Fish Handling Procedures – The following detailed chronology of fish handling procedures from the point at which the fish is removed from the holding system, through the completion of the ultrasound procedure, was derived from Table 3.3 and from the studies reviewed. The minimum information necessary for reporting would be as follows: (1) holding system dimensions, water volume, and stocking rate; (2) whether or not the fish were starved (purged) and how long feed was withheld; (3) equipment used to move the fish from the holding system to the container used for ultrasonography (e.g., fish hauler, type of net, baskets,

or if fish was moved by hand); (4) whether or not the fish was alive or dead (including time of postmortem diagnosis) before or during ultrasonography; (5) whether or not anesthesia was used, including the chemical name and dose, and how long the anesthetic was effective if the fish was anesthetized; (6) dimensions, or water volume, of container used during ultrasonography (if different from the holding system), and how long the fish was held in this container; (7) whether or not the fish was physically restrained and, if so, how this was done; (8) whether the fish was maintained completely or partially submersed in water, or if it was removed from the water; (9) explicit descriptions of fish position (orientation), that is, in ventral, dorsal, or lateral recumbency; (10) explicit description of the scanning region and procedure, including probe position and movement with respect to standard external anatomical features for the species; (11) an illustration, such as a digital photograph or schematic representation, showing the positioning of fish and probe during the ultrasound procedure; (12) duration of the scanning procedure per fish; (13) equipment used to move the fish from container to a recovery system; and (14) method or equipment for final transport of fish after scanning to the holding system.

Greatest utility would come from fish handling that consistently integrates procedures to minimize stress, such as retaining the fish in water, using the water as an ultrasound transmission medium, and using unrestrained, unanesthetized, submersed fish, such as the procedures recently described for imaging of channel catfish ovaries (Guitreau et al. 2012). The use of ultrasound imaging in reproduction studies can be elevated to a higher level of noninvasive procedures by reporting and standardizing minimal handling procedures for specific groups of fish, giving special considerations for the biological diversity range illustrated in these studies. If the full utility of ultrasound imaging as a noninvasive tool is to be exploited, it will be necessary to

report equipment-specific information, settings, and the detailed handling procedures used before, during, and after ultrasound imaging. Attention should also be given to developing procedures suitable for commercial-scale work outside of the laboratory.

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Chapter 4
Development of a Standardized and Systematic Approach to Interpretation of Ovarian Ultrasound Images Captured During Monitoring of Hormone-Induced Spawning of Channel Catfish *Ictalurus punctatus*

Artificial spawning of fish is a process that involves reproductive conditioning and selection of hormone-induced oocyte maturation and ovulation, collection of unfertilized eggs, fertilization, incubation, and rearing of larvae to fry for culture. The goal of artificial spawning in aquaculture is to increase control over reproduction for production of market-sized fish, and for genetic improvement, for instance through selective breeding, hybridization or ploidy manipulation, leading to superior production traits such as increased meat yield, disease resistance, or feed conversion efficiency. Channel catfish *Ictalurus punctatus* broodstock are generally conditioned for reproduction in earthen ponds before capture and selection of females for hormone injection based on external morphology of secondary sexual characteristics as judged through the experience of the fish culturist and trained farm personnel (Phelps et al. 2011). The general guidelines for desirable external characteristics include "a full, well rounded abdomen," or "potbelly," ' which should extend to the genital orifice, and which should be "soft and palpable" with "flat" or "slightly depressed" genitals "of a reddish color" (Lee 1981). These external characteristics are associated with potential for hormone-induced spawning and production of viable eggs, and they constitute the main mode of selection of females for reproduction research, and for commercial production of C x B hybrid (channel catfish female x blue catfish male *I. furcatus*) (Bosworth et al. 2005; Phelps et al. 2011).

External morphological features were placed in a qualitative classification system of "good," "fair," and "poor," for systematic analysis alongside quantitative morphometric measurements of total length, girth, body width, and ratios (such as length-to-width, length-to-girth, length-to-

weight) as broodstock criteria for selection of female channel catfish for hybrid production (Phelps et al. 2011). The length-to-girth and length-to-weight ratios were not useful in assessing ovarian development, but the subjective classification of broodstock characteristics by experienced individuals and the length-to-width ratio significantly affected the ability to predict spawning success (Phelps et al. 2011). Ovarian reproductive assessment for artificial spawning may also be based on egg catherization for examination of egg size, and examination of the position of the nucleus (Markmann and Doroshov 1983; Stoeckel 2000; Phelps et al. 2011). But catheter use and fish handling can physically injure the ovary, and interrupt the reproductive process (Newman et al. 2008; Phelps et al. 2011). In contrast to these methods, minimally stressful fish handling and ultrasonography procedures have been developed for use in indoor and outdoor holding systems (e.g. recirculating tank systems, raceways, or ponds) and demonstrated high potential for obtaining useful imaging data on the reproductive condition of the channel catfish ovary (Guitreau et al. 2012).

Ultrasonography is a technology comprising equipment and software that use sound emission (frequencies > 20,000 hertz) and reception to create images of internal anatomy based on positioning of the probe (the part of the ultrasound unit that emits and receives ultrasound waves) with respect to external anatomy for routine disease and reproduction diagnostics in humans and farmed animals. Ultrasonography has been used in fisheries and aquaculture in 21 fish species in 27 studies of sex identification and development of reproductive indices from 1983 to 2010 (Novelo and Tiersch 2012). In these studies, control settings were not reported, and variable fish handling and imaging techniques even within the same species of similar age fish indicated a

need for standardization that would enable experienced or novice users of the technology to replicate and cross-reference studies for comparison of findings (Novelo and Tiersch 2012).

Ultrasonography is considered advantageous for its physically non-intrusive nature (as opposed to the use of catheters or biopsy procedures for histology). The use of this technology incorporates challenges such as protocol development, technical training, variable machines, software, and control setting features. This study was designed to generate a method for assessment of broodstock reproductive condition by means of systematic observations and standardized recording and reporting to provide clear procedures and to address these technology challenges. These procedures represent standardization of mechanisms that realize the potential for maximizing the utility of this technology in fish reproduction.

Ultrasound imaging was used in this study to form part of a reproductive technology platform in hormone-induced spawning of channel catfish conditioned in heated ponds before the natural spawning season, and in ambient-temperature ponds during the natural spawning season. The goal was to devise a method for ultrasound image interpretation of the reproductive state of the ovary during monitoring of hormone-induced spawning. The objectives were to: (1) evaluate spawning rate, spawning latency, and egg fertilization for characterization of ultrasound images; (2) assess the use of "gonadal" or "gonad and gametic" viewing areas during monitoring of the ovary; (3) develop a systematic method for interpretation of ultrasound images of the channel catfish ovary. This study identified the use of ultrasonography from a "gonadal" or "gonad and gametic" viewing areas for assessing reproductive state, and defined images of oocytes undergoing hydration and maturation, mass oocyte retention, and atresia.

Methods

Hormone-Induced Spawning and Fertilization. Stocking, Thermal Conditioning, and Hormone-Induced Spawning. Channel catfish broodstock (1.5 ± 0.3 kg; 50 ± 3 cm) were purchased from Haring Fish Farms Inc. in Wisner, Louisiana, and stocked in December 2004 in 0.04-ha earthen ponds for six spawning trials in 2005 at the Aquaculture Research Station, Louisiana State University Agricultural Center in Baton Rouge, Louisiana (lat 30°22'1.93" N, long 91°10'13.30" S). Fish were conditioned for reproduction using thermostatically controlled geothermal water (Hall et al. 2002) before the natural spawning season for the first four spawning trials, and using ambient temperatures during the natural spawning season for the last two spawning trials (Table 4.1, Figure 4.1).

Initial temperatures (average ± SD) of the two ponds stocked in January (19 ± 1 C), and of the two ponds stocked in February (15 ± 4 C) were increased by 2 C/day until 28 C was reached, and water was kept at this temperature for 3 to 5 weeks, after which heating ceased and fish were captured by seine (Figure 4.1). Water temperature was recorded throughout thermal conditioning for each spawning trial by data loggers (HOBO data logger; Onset Computer Corporation, Pocasett, MA, USA). The temperature data collected were used to calculate the thermal conditioning profile expressed as degree days, the sum of the mean daily temperature above 21 C (Pawiroredjo et al. 2008).

Table 4.1 All 0.04-ha ponds were stocked with channel catfish broodstock in December, 2004 for the six artificial spawning trials in 2005 listed chronologically according to the date. Four ponds (G10, G9, G8, and G6) were heated in sets of 2 using manipulated geothermal water (36 C) for spawning trials before the start of the natural spawning season. Two ponds (G1, G2) were maintained at ambient water temperature for spawning trials during the natural spawning season. The total weight (kg ± SD) and total length (cm ± SD) of the catfish (n = 12 females/spawning trial) monitored using ultrasonography were listed below.

Spawning Trial	Date	Pond	Sexes* Stocked	Heating Started	Captured by Seine	Total Weight	Total Length
I	February 9 - 12	G10	30 F, 10 M	January 12th	February 7th	1.5 ± 0.2	52 ± 2
II	February 21 - 24	G9	30 F	January 12th	February 18th	1.4 ± 0.2	51 ± 2
III	March 29 - April 1	G8	30 F, 10 M	February 22nd	March 25th	1.4 ± 0.2	50 ± 2
IV	April 5 - 11	G6	30 F	February 22nd	April 1st	1.5 ± 0.2	51 ± 3
V	May 31 - June 2	G1	30 F	Ambient	May 27th	1.6 ± 0.4	53 ± 4
VI	June 14- 17	G2	30 F, 10 M	Ambient	June 10th	0.8 ± 0.2	43 ± 3

*F, female; M, male.

After fish were captured, they were held in a wet laboratory recirculating system for 2 to 4 d prior to spawning trials (Table 4.1, Figure 4.1). Twelve females were selected for each spawning trial based on external morphology (e.g. roundness of the belly) (Lang and Tiersch 2007). These females were injected with a single dose of 100 µg of luteinizing hormone-releasing hormone analog/kg (Syndel International Inc., Canada) of fish. The injected fish were placed in eight 80-L fiberglass tanks (35 X 50 X 58-cm tanks, with 28-cm standpipes) for observation and ultrasound image monitoring. Four tanks held a male-female pair, and four tanks held a female-female pair during each time period. The spawning latency of each female was defined and recorded as the time (hours) between hormone injection and ovulation (observed egg expulsion) or stripping (manual collection) of eggs (Bates and Tiersch 1998).

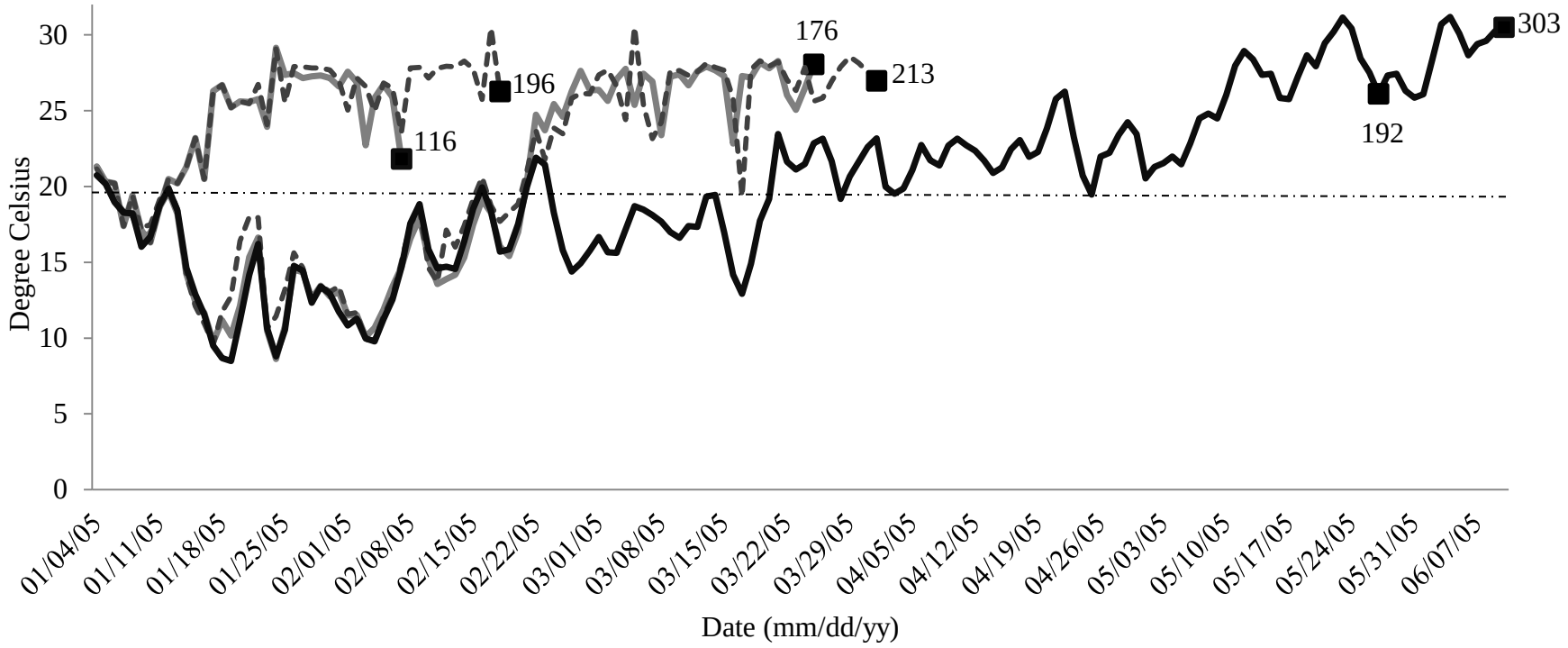


Figure 4.1 Temperature profiles of earthen ponds (0. 04-ha) used for reproductive conditioning of channel catfish broodstock during six spawning trails in 2005. The solid black line represented the ambient temperatures. The threshold temperature of 21 C is indicated by the dashed-dotted straight line. All other lines represent the temperature of heated ponds. Fish captured by seine for each spawning trial are indicated by squares. The sum of the difference of the mean daily temperature and 21 C (degree days) was calculated for quantifying thermal reproductive conditioning from the starting date to the date of capture by seine for each group of fish in heated ponds before the natural spawning period (116, 196, 176, 213 degree days) and for each group of fish in ambient temperature ponds during the natural spawning period (solid black line 192, 303 degree days).

The spawning rate for each spawning trial was calculated as the number of females producing viable eggs divided by the total number of hormone-injected females multiplied by 100 to obtain a percentage.

Fertilization Experiment Design – The average number of eggs in a monolayer sample (~ 115 eggs) in the bottom of a 100-mL tri-corner plastic beaker (Thermo Fisher Scientific Inc., Suwanne, GA, USA) was estimated by averaging the count of the number of eggs in three different monolayer samples from each female. Twelve monolayer samples of eggs from each female were fertilized with sperm from three males (four replicates per male), except for spawning period VI when one male was used (Table 4.2). Fertilization was estimated by counting the number of eggs that progressed to neurulation, the stage of embryonic development when the neural tube is formed and is visible as a line when illuminated from below with a light source (Pawiroredjo 2004). The fertilization rate for each of the 12 monolayer samples for each female was calculated as the number of neurula divided by the average number of eggs in the female monolayer sample multiplied by 100 to obtain a percentage.

Statistical Analysis. The generalized linear mixed model (GLIMMIX) procedure of the Statistical Analysis Software system version 9.3 for Microsoft® Windows® (SAS Institute Inc., 2012, Cary, NC, USA) was used to perform separate tests for differences in spawning latency, spawning, and fertilization rate. Spawning latency and spawning rate were statistically analyzed for the six spawning trials combined because all fish underwent the same procedures for inducing ovulation, oocyte maturation and egg collection but, the experimental design for assessing fertilization was different during the spawning trials. The design (12 monolayers of

eggs/female fertilized by 3 males with four replicates each male) was the same for spawning trials III, IV, and V; fertilization data of these 3 spawning trials were included in statistical analysis. In the remaining spawning trials different sperm concentrations were used (Trial I), dilution data were available but not sperm concentration (Trial II), and one male was used (Trial VI) during fertilization, (Table 4.2); therefore the fertilization data collected during these three trials were not included in the statistical analysis.

All of the statistical models used included three fixed variables, and time period of the spawning trial as a random variable (i.e., a block). The first fixed variable was temperature; that is, pond water temperature was either: (i) heated using geothermal water provided by thermostatic control, or (ii) ambient water temperatures. The second fixed variable was pond sex ratios; that is, the ponds were either stocked with male and female fish (1:3 mixed sex), or stocked only with females. The third fixed variable was tank pair sex ratio; that is, fish were stocked either as a female-female pair in four tanks, or as a male-female pair in four tanks in an 8-tank recirculating system during hormone-induced spawning. Two additional fixed variables, male and sperm concentration were included in the models evaluating for differences in fertilization in spawning trials III, IV, and V (Table 4.2). A number of link (whole equation transformations) and probability distributions were possible for these data, and the combination of link and probability distribution (with a chi-square/degree of freedom fit statistic closest to a value of 1) was selected for each of the models. For spawning latency, the combination of log link and Poisson distribution was optimal, and for spawning and fertilization, the combination of logit link and binomial distribution exhibited the best fit.

Table 4.2 Biological characteristics of males used for artificial fertilization during six spawning trials.

Spawning Trial	Male Identification	Total Length (cm)	Total Weight (kg)	Testis Weight (kg)	Initial Sperm Concentration (cells/mL)	Sperm Motility (%)
I	CCFH05M29	51	1.4	5.32	2.70×10^8	40
I	CCFH05M30	58	2.0	9.29	1.50×10^8	60
I	CCFH05M31	55	1.8	11.51	2.20×10^8	90
I	CCFH05M32	53	1.4	6.26	1.60×10^8	10
I	CCFH05M33	54	1.4	4.68	1.70×10^8	40
II*	CCFH05M34	53	1.3	5.59	-	30
II*	CCFH05M35	57	1.8	4.67	-	20
II*	CCFH05M36	53	1.4	6.62	-	40
II*	CCFH05M37	52	1.3	1.53	-	10
II*	CCFH05M38	54	1.3	6.86	-	10
III**	CCFH05M44	52	1.4	4.53	2.85×10^8	40
III**	CCFH05M45	54	1.5	5.18	8.76×10^8	50
III**	CCFH05M46	55	1.6	7.67	1.62×10^8	50
IV**	CCFH05M47	50	1.5	6.62	2.49×10^8	40
IV**	CCFH05M48	52	1.6	12.29	1.26×10^8	70
IV**	CCFH05M49	52	1.7	6.82	9.16×10^8	50
V**	RIOBCFH05M04	75	4.6	-	3.15×10^8	60
V**	RIOBCFH05M05	75	5.1	8.54	3.21×10^8	50
V**	RIOBCFH05M06	68	3.4	4.84	3.68×10^8	30
VI	CCFH05M71	56	1.7	10.81	3.32×10^8	50

*Sperm was diluted into 1 g testis: 5 mL HBSS; this was diluted into aliquots of 1:10, 1:20, and 1:40; sperm concentration not available.

**Sperm was diluted from initial concentration to 10^6/mL, 10^7/mL, and 10^8/mL.

To evaluate the effect of temperature and pond sex ratio, we used two statistical models because ponds stocked with male and female fish only appeared in one of the two temperature treatments. The first model tested for differences without the temperature variable to

be able to evaluate the effect of sex ratio, and the second model tested for differences without the sex ratio to be able to evaluate the effect of thermal conditioning. Differences were considered significant at $P < 0.05$ for all statistical tests.

Use of "Gonadal" or "Gonadal-gametic" Viewing Areas. Fish Handling, Imaging, and Settings. Ultrasound images of the ovary and oocytes were obtained using a portable ultrasound unit, the Classic TelaVet 1000™ Veterinary Digital Ultrasound Module (Telemed UAB, Vilnius, Lithuania) and a waterproof linear probe (model LV7.5/60/96Z). Ultrasound imaging followed the 0initial fish handling and imaging procedures reported previously in detail (Guitreau et al. 2012). Briefly, during imaging, the fish were free-swimming within the tank, with constant aeration and water flow and no anesthesia or physical restraint (Guitreau et al. 2012). The ovary was viewed with the tip of the probe placed in the water pointing towards the base of the tank, and the cable end of the probe beside the fish. The probe was moved alongside the left lateral abdominal surface of the fish, closer or further from the skin surface to adjust the focus setting and record images of the ovaries. Using this consistent handling technique, paired ultrasound videos were recorded during spawning trials I to III in Audio Video Interleave (AVI) format from the time of injection designated as 0 h through 63 to 81 h producing a total of 646 videos, and during spawning trials IV to VI at the time of injection and at the time of egg collection (and at three additional times during spawning trial V) producing a total of 182 videos (Table 4A.1). For each pair, the first video was recorded using settings which provided a gonadal view ("wide angle"), and the second video was recorded using settings which provided a gonadal-gametic view ("close-up") (Table 4.3). The videos were captured within 1 to 2 min of each other.

Table 4.3 The ultrasound control settings used for capturing videos of channel catfish ovaries during hormone-induced spawning (brief definitions from the Echo Wave Ultrasound Software Operation Manual, Revision 2.7, 2006). The settings were titled 'Both Ovaries' for the gonadal view, and 'Ovary' for the gonadal-gametic view. These settings were stored in the pre-set option for documentation, for ensuring consistency in use of ultrasound controls, and for quick retrieval.

Controls	Definition	'Both Ovaries'	'Ovary'
Probe Frequency	Selects operating frequency from the multi-frequency probe	5 MHz	8 MHz
Transmit	Controls focusing at a depth (mm) location during transmission	35 mm	10 mm
Depth	Sets the scanning depth	110 mm	80 mm
Image Enhancement	Makes edges and border areas more visible	2	2
Dynamic Range	Controls image contrast	61 dB	56 dB
Frame Average	Sets how many frames will be averaged to lower *noise level	4	4
Scroll	Shifts displayed scanning image from selected depth value	0 mm	0 mm
Zoom	Sets the zoom ratio	100 %	100 %
Power	Acoustic power of ultrasound beam	82 %	90 %
Gain	Sets voltage amplitudes and echo brightness	82 %	70 %
Time-Gain Compensation (TGC)	Horizontal sliders that adjust the gain at specific depths	**TGC	
Rejection	Ultrasonic signal rejection to reduce noise visibility	0	0

*Noise: unwanted background sound artifacts that can arise from within the scanner, the subject, the interaction of ultrasound with tissue structure, or from electronic interference from nearby equipment (Toal 1996, Hill 1989).
**TGC was adjusted along the depth settings of ultrasound penetration at 0, 27, 55, 82 and 110 mm for 'Both Ovaries' and at 0, 20, 40, 60 and 80 mm for 'Ovary'.

Formatting for Ultrasound Image Figures. The Echo Wave software version 3.60 (Telemed UAB, Vilnius, Lithuania) of the ultrasound unit was used to capture still images in Windows bitmap (BMP) format from each AVI video. The BMP images included a black background on the sides, a white sidebar on the right, and the viewing area (i.e. the ultrasound image of the water and the fish cross-section) (Figure 4.2). These images were edited for figure illustrations. Images of the gonadal view were cropped using the Microsoft Word 2010 picture formatting tool by dragging the cursor to 90 mm (i.e. 0 - 90 mm of the image was used).

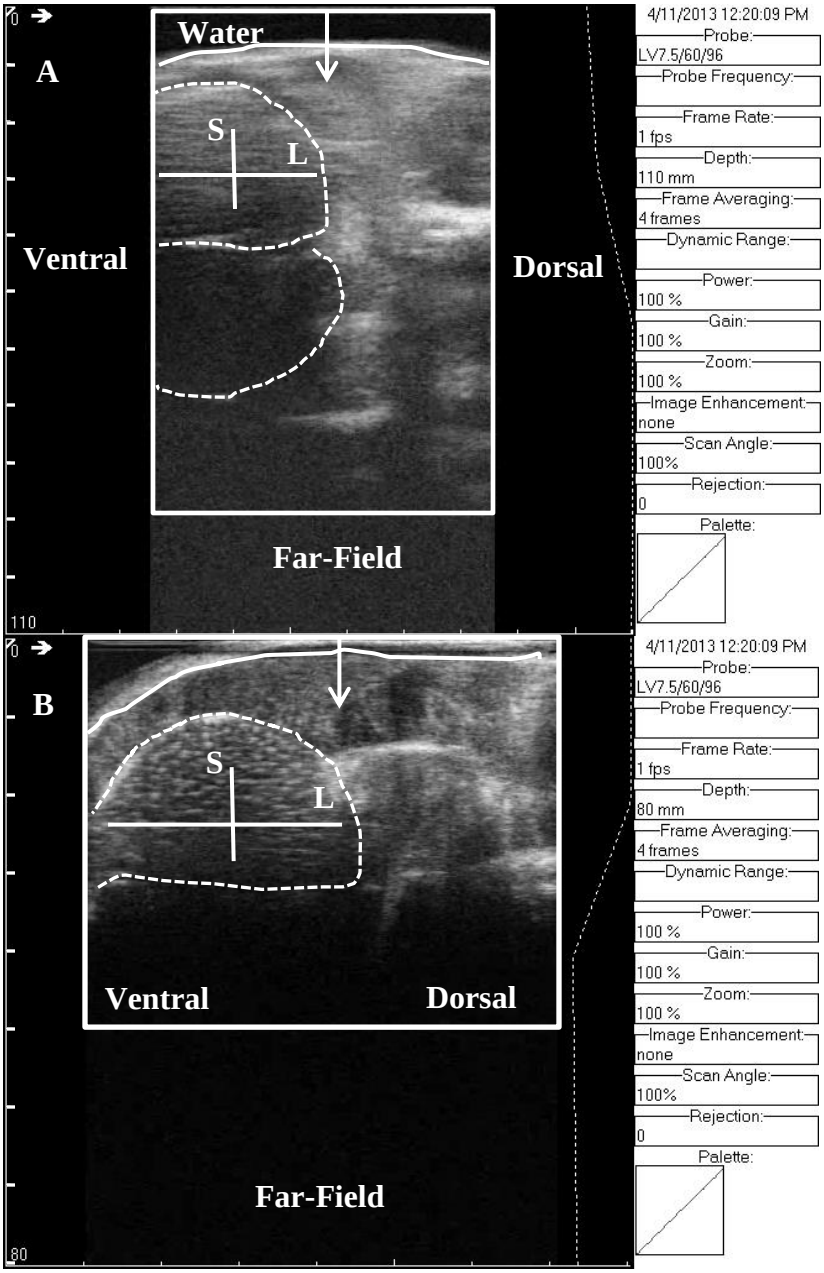


Figure 4.2 The ultrasound image (bitmap image format) of ovarian cross-sections as they appeared when viewed as gonadal (A) or gonadal-gametic (B) images. The markings on the left of the images indicate intervals of 10 mm depth and the date of still image capture with vestige (i.e. inaccurate) ultrasound controls settings from the still image capture procedure are listed on the right of the image. The white box outlines the area used in figure illustrations. The solid white line demarcates the skin, the dashed line define the ovarian cross-section, the white lines perpendicular to each other within the ovary depict the long (L) and short (S) axes, and the arrow in the white box indicates the direction of ultrasound emission from the probe.

Images which displayed the gonadal and gametic view were cropped in the same manner to 50 mm (i.e. 0 - 50 mm of the image was used). The left and right sides of the images were cropped to the size of the viewing area, and the remaining image was resized relative to the original image.

Development of a Standardized and Systematic Approach for Ultrasound Image Interpretation. Biology and Ultrasonography. The development of this approach was based on the following assumptions regarding channel catfish spawning and ultrasonography. The first assumption was that the biological processes observed during ultrasonographic monitoring of hormone-induced spawning were the outcome of multiple interdependent effects. Key examples of these interdependent effects were temperature, the primary environmental factor controlling reproductive development and manipulation of channel catfish spawning (Davis et al. 1986; Lang et al. 2003), and the reproductive state of the gonad and gametes at the time of hormone-injection (Mylonas et al. 2010). The second assumption was that the ultrasonographic processes involved in the real-time viewing of the ovary, recording of videos (AVI), capture of still images (BMP), and illustrations were the outcome of multiple factors and procedures. For example, one essential factor was use of initial procedures developed for consistent fish handing and obtaining imaging data (Guitreau et al. 2012). The collection of imaging data with use of ultrasound controls at constant settings ensured that no artifacts arising from the use of inconsistent or variable use of individual control settings would occur. This provided a level field for comparison of multiple ultrasound images during the monitoring of individual fish and groups of fish in the six spawning trials.

Therefore, the standardized and systematic approach developed for interpretation of ultrasound images was comprised of biological insight and ultrasonographic processes. The biological insight consisted of: (i) the composition and location of the ovary, (ii) known processes occurring during hormone-induced spawning, (iii) the temperature history, the time of the year, and the sampling site location, which were indicative of thermal reproductive conditioning and readiness to spawn with reference to degree-day guidelines, and (iv) the biological outcomes of hormone-induced spawning (egg and fertilization profile). The ultrasonographic processes consisted of : (i) use of consistent fish handling and imaging techniques; (ii) use of ultrasound imaging technology features, including characterizing structures in the echo image display, use of consistent anatomical markers in images, and identification of ultrasound image artifacts; (iii) use of gonadal-gametic viewing areas for identifying changes in the shape, size, and echogenicity (i.e. the echogenic morphology or pattern caused by ultrasound-tissue interaction) of the ovary and oocytes. This systematic approach to interpretation of ultrasound images required inferences from different observations that would comprise the ultrasonographic assessment of the ovarian reproductive state, as opposed to one isolated step (e.g. interpretation based solely on the image displayed).

Results

Hormone-Induced Spawning and Fertilization. **Spawning Latency, Spawning Rate, Neurulation Rate.** The average spawning latency (the time between hormone-injection and egg collection) ranged from 32 h to 57 h, and the spawning rate (the number of fish producing viable eggs/total number of hormone-injected females) ranged from 17 to 42% for spawning trails I to VI, and the neurulation rate ranged from 53 to 73% for spawning trials III, IV and V (Figure 4.3).

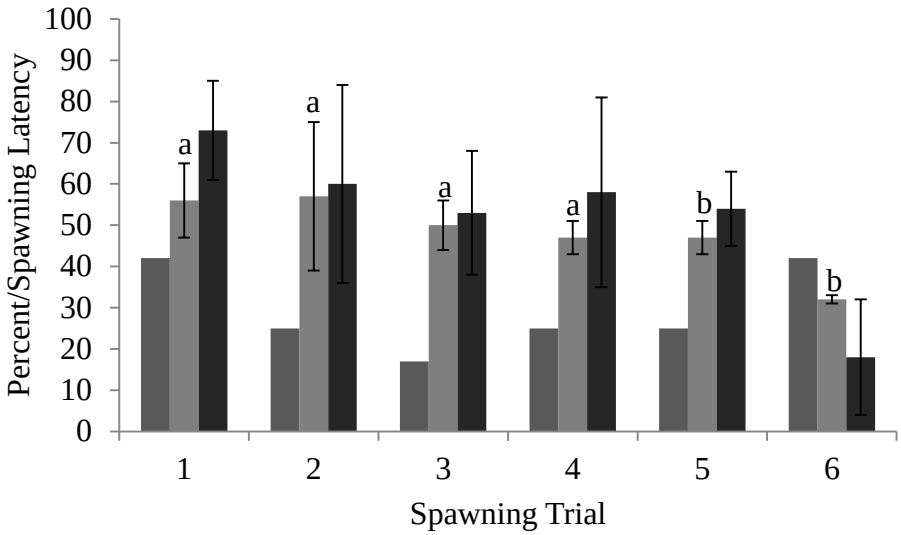


Figure 4.3 Spawning rate (the number of females which produced viable eggs/number of hormone-injected females) indicated by the first bar in each trial (horizontal axis) ranged from 17% (Trial 3) to 40% (Trial 1). The average (±SD) spawning latency (the time from hormone-injection to collection of viable eggs) indicated by the second bar in each trial (horizontal axis) ranged from 32 (±1) (Trial 6) to 57 (±18) h (Trial 2). The average (±SD) neurulation indicated by the third bar in each trail (horizontal axis) ranged from 18 % (±14) (Trail 6) to 73 % (±12) (Trial 1). The use of heated or ambient temperature had a significant effect ($P < 0.05$) on spawning latency (denoted by differences in small letters on the spawning latency bars), but it did not have a significant effect on spawning rates, or fertilization rates.

Tests on the effects of all-female or male-female ponds, heated or ambient water temperatures, female-female or male-female tank pairing during hormone-induced spawning, sperm concentration, and male used on spawning latency, spawning rate, and fertilization were not significantly different ($P > 0.05$) for the spawning trials tested (see Table 4.4) except for one test. The difference was significant ($P < 0.05$) for the effect of temperature (heated and ambient) on spawning latency. Female-female or male-female tank pairing had no effect on latency regardless of thermal conditioning (Table 4.4).

Table 4.4 There were no significant differences ($P > 0.05$) in spawning rates and fertilization among heated or ambient-temperature ponds , the use of all-female or male-female pond stocking (Pond Sex), pairing of fish in male-female or female-female pairings (Tank Pair), sperm concentration, or males used in spawning trials tested. There was no significant difference in spawning latency for all the effects tested, except for Temperature ($P < 0.05$).

Fixed Effects	Num DF	Den DF	F Value	Pr > F
Spawning Latency (Spawning Trials I to VI) without the pond sex variable				
Temperature	1	4	10.4	0.03
Tank Pair	1	3	0.42	0.56
Tank Pair*Temperature	1	3	0.11	0.77
without the temperature variable				
Pond Sex	1	4	0.6	0.48
Tank Pair	1	3	0.13	0.75
Pond Sex* Tank Pair	1	3	1.14	0.36
Spawning Rates (Spawning Trials I to VI) without the pond sex variable				
Temperature	1	4	5.77	0.07
Tank Pair	1	3	0.09	0.78
Tank Pair*Temperature	1	3	0	0.99
without the temperature variable				
Pond Sex	1	4	3.25	0.15
Tank Pair	1	3	0.15	0.73
Pond Sex* Tank Pair	1	3	0.03	0.87
Fertilization (Spawning Trials III, IV, and V) without the temperature variable				
Pond Sex	1	1	6.2	0.24
Tank Pair	1	1	0.01	0.95
Pond Sex* Tank Pair	1	1	27.45	0.12
Sperm Concentration	3	3	2.85	0.21
Pond Sex* Sperm Concentration	3	3	2.84	0.21
Tank Pair* Sperm Concentration	3	3	0.88	0.54
Pond Sex* Tank Pair *Sperm Concentration	3	3	1.51	0.37
without the pond sex variable				
Temperature	1	1	0.01	0.93
Tank Pair	1	1	9.03	0.20
Tank Pair*Temperature	1	1	0.2	0.73
Sperm Concentration	3	3	0.76	0.59
Temp*Sperm Concentration	3	3	1.49	0.37
Tank Pair*Sperm Concentration	3	3	0.43	0.75
Tank Pair*Temperature*Sperm Concentration	3	3	0.24	0.87
Effect of Males Used	8	78	1.43	0.20

Use of "Gonadal" or "Gonadal-gametic" Viewing Areas. **The Gonadal View.** Ultrasonography from the gonadal view generated a broad discernible anatomical view of the two ovaries (Figures 4.2A, 4.4, 4.5). The ovary nearest the probe in the near-field view (0 – 40 mm) view was clearer than the adjacent ovary in the far-field view (40 – 110 mm), with the ovary furthest from the probe appearing homogenous dark-grey to black (Figure 4.2A). The ovarian walls delineated the area of the ovarian cross-section in the ultrasound image. The ovarian walls separating the cross-section of the two ovaries appeared as a greyish line that was hyperechoic to the surrounding ovarian tissues and demarcated the borders of the adjacent ovaries. The gonadal view produced a homogenous light or dark-grey shaped appearance of the ovary, but this echogenic pattern was not useful for monitoring oocytes.

The Gonadal-Gametic View. Ultrasonography from the gonadal-gametic view generated a complex echogenic pattern caused by the appearance of discernible oocyte structures within the ovary displayed in the near-field view (0 – 40 mm), while the remaining display (40 – 80 mm) appeared black (anechoic); individual oocytes with demarcated boundary zones were identifiable within the ovarian walls (Figures 4.2B, 4.6, 4.7). The first oocyte feature was the white to bright grey ultrasound reflection within the oocyte. This feature was especially prominent in the near-field view of the image closer to the probe. As the ultrasound waves penetrated to oocytes deeper within the ovary and approached the ovarian wall furthest from the probe, the intensity of the white-grey reflection (i.e. the echogenicity of the oocyte) decreased.

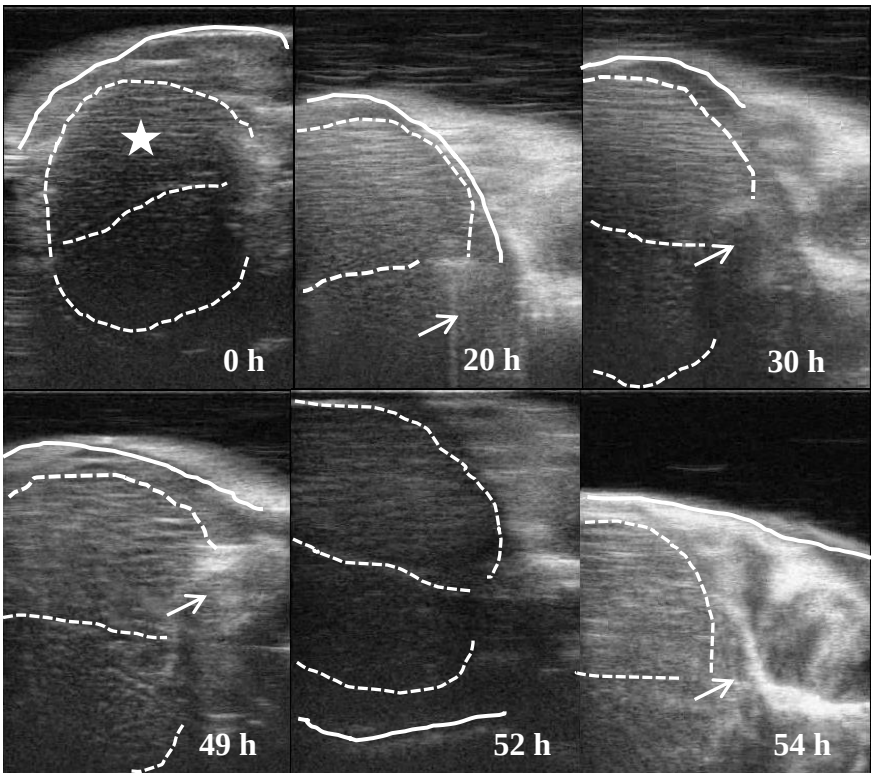


Figure 4.4 Representative ultrasound images (Fish ID F70) of channel catfish ovaries from which viable eggs were collected. These images were captured using a gonadal view which provided deeper ultrasound penetration but lower resolution, resulting in a broad view of the paired ovaries but no clear image of the oocytes at the time of injection (0 h) up to before (52 h) and after (54 h) egg collection. Differences in the distances of the probe to the skin and the positioning of the ovary resulted from the movement of the free-swimming catfish within the tank of the recirculating system used for artificial spawning. Solid white lines = skin; dashed lines = ovaries; the star indicates the position of the ovary nearest the probe; the arrows point at the areas of ultrasound image artifacts created during fish movement.

The second oocyte feature was the dark ring-like structure that surrounded the hyperechoic center of the oocyte. The complete image of the oocyte cross- section was comprised of the white or bright grey (hyperechoic) structures, graduating into darker (hypoechoic) structures which demarcated the boundary zone to adjacent oocytes.

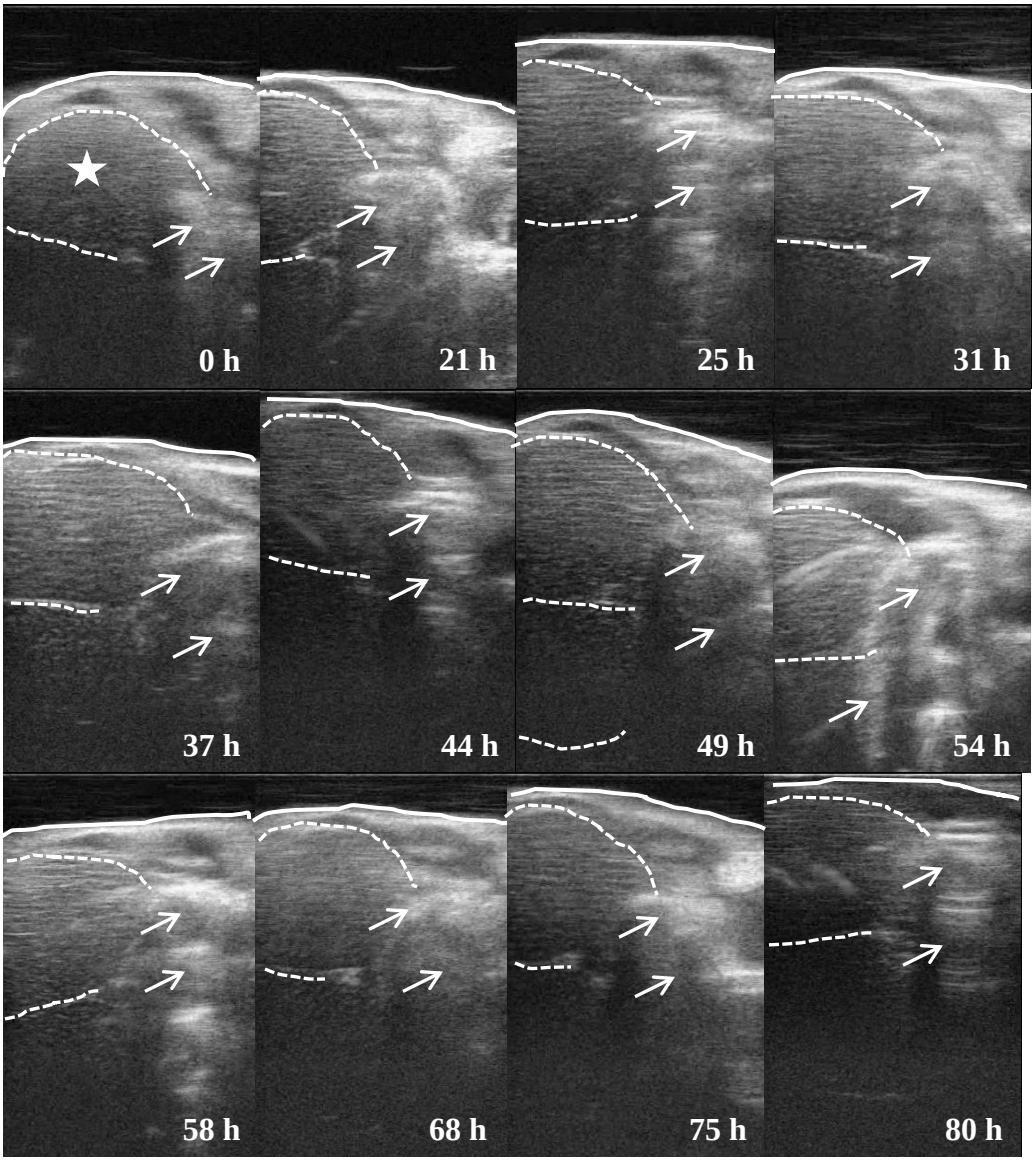


Figure 4.5 Representative ultrasound images (Fish ID F49) of channel catfish ovaries from which no eggs were collected. These images were captured using a gonadal view which provided deeper ultrasound penetration, lower resolution, a view of the paired ovaries, but no clear image of the oocyte from the time of injection (0 h) up to 80h. Differences in the distances of the probe to the skin and the positioning of the ovary resulted from the movement of the free-swimming catfish within the tank of the recirculating system used for artificial spawning. Solid white lines = skin; dashed lines = ovaries; the star indicates the position of the ovary nearest the probe; the arrows point at the areas of ultrasound image artifacts created during fish movement.

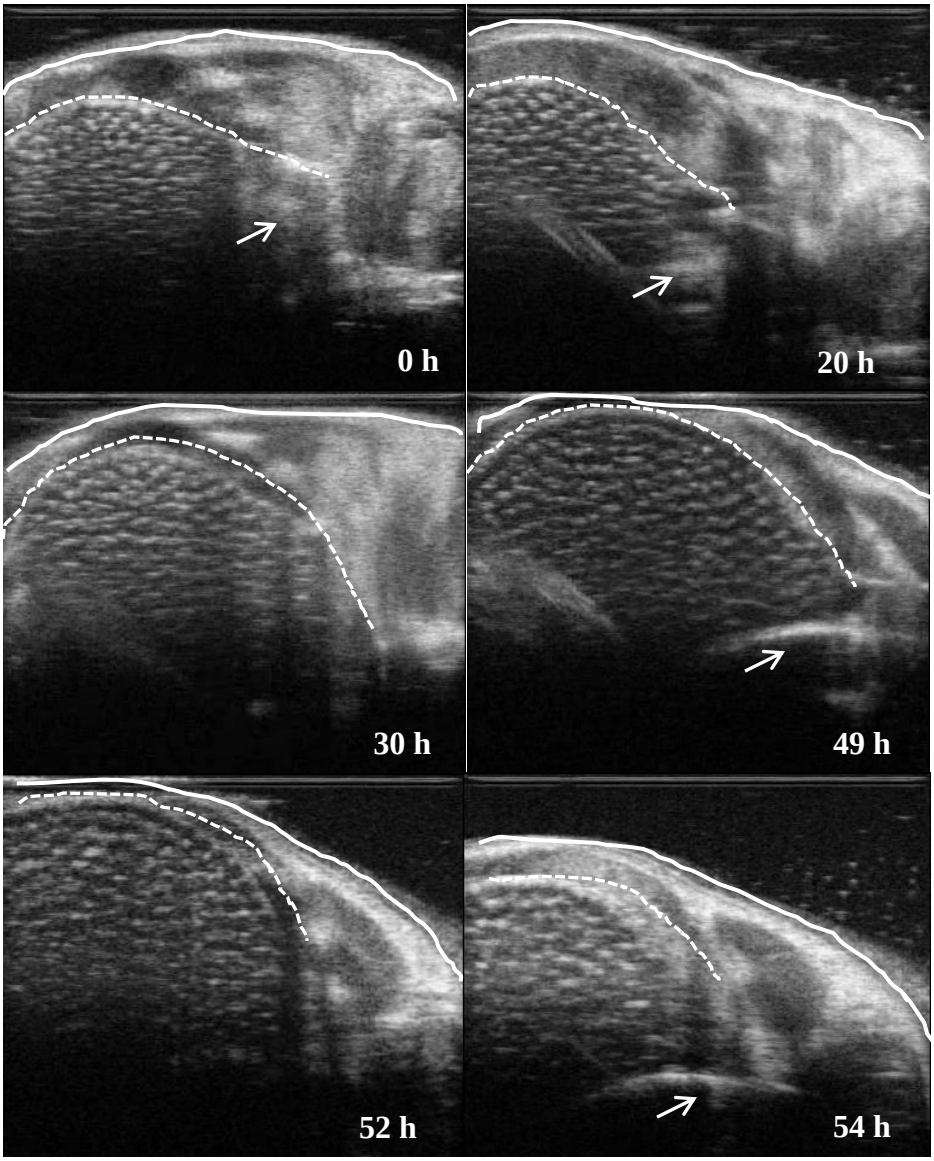


Figure 4.6 Representative ultrasound images (Fish ID F70) of the ovary nearest the probe (denoted by the star in 4.4). These images were captured using a gonadal-gametic view which provided images with less ultrasound penetration but higher resolution, thereby resulting in echogenic appearance of the oocytes encased within the ovary from the time of injection (0h) up to before (52 h) and after (54 h) egg collection. Solid white lines = skin; dashed lines = ovary; the arrows point at the areas of ultrasound image artifacts created during fish movement.

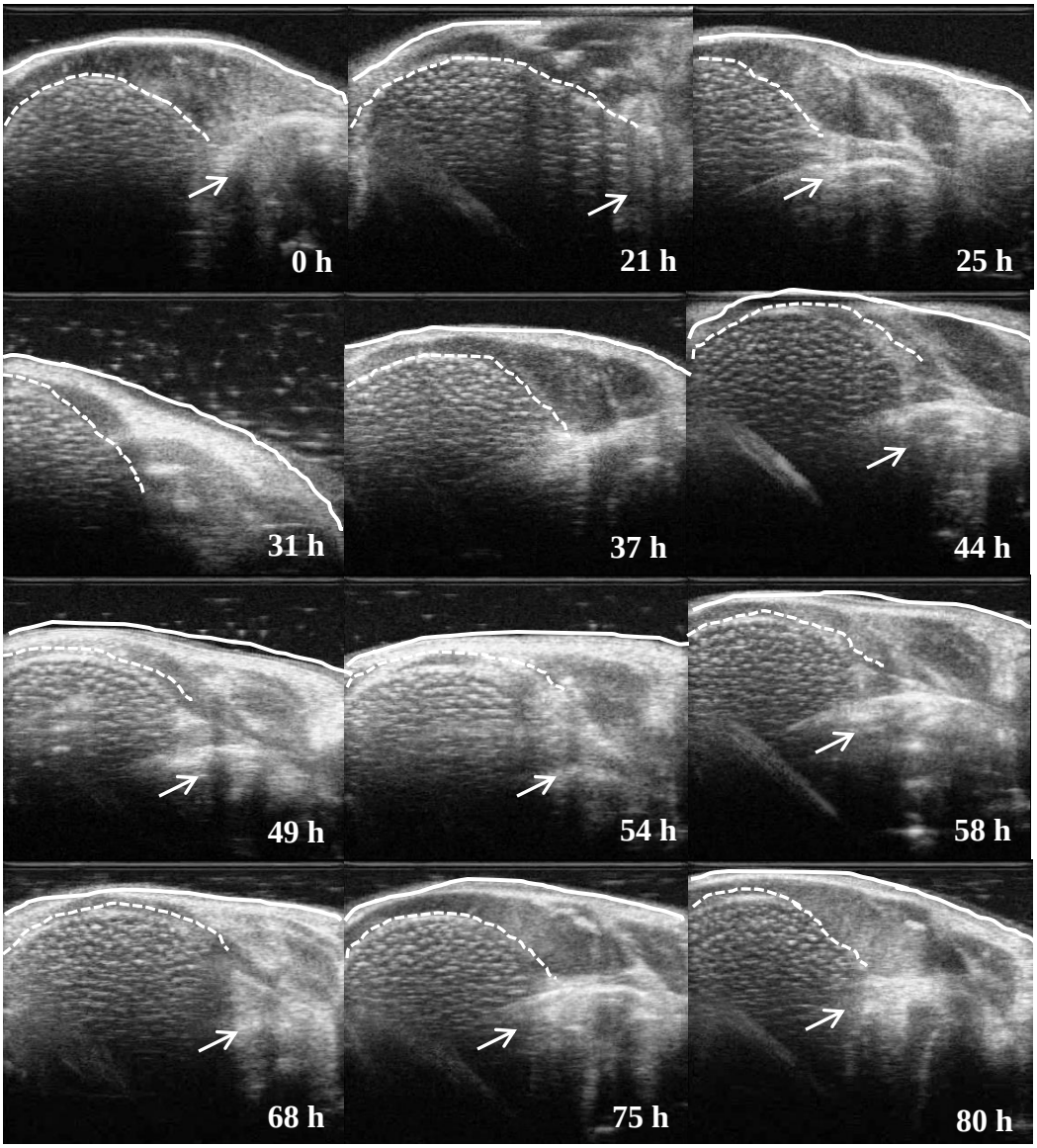


Figure 4.7 Representative ultrasound images (Fish ID F49) of the channel catfish ovary nearest the probe (denoted by the star in 4.5) from which no eggs were collected. These images were captured using a gonadal-gametic view which provided images with higher resolution and the distinct appearance of oocytes encased within the ovary from the time of injection (0h) up to 80h. Differences in the distances of the probe to the skin and the positioning of the ovary resulted from the movement of the free-swimming catfish within the tank of the recirculating system used for artificial spawning. Solid white lines = skin; dashed lines = ovaries; the arrows point at the areas of ultrasound image artifacts created during fish movement.

Development of a Standardized and Systematic Approach for Ultrasound Image Interpretation. The design of the standardized and systematic approach for ultrasound image interpretation during monitoring of hormone-induced spawning was based on integration of two fundamental building blocks – the biological and ultrasonographic insights (Figure 4.8).

Biological Insight. (i) Location and Composition of the Ovary – In teleosts, the ovary consists of oogonia, oocytes and their surrounding cells, supporting tissue, vascular and nervous tissues, and in cyclical breeders the ovary can vary considerably in appearance at different times during the cycle (Nagahama 1983). The ovary is enclosed in a tunica albuginea (referred to in this study as the ovarian 'wall') which consists of fibrous connective tissue and smooth muscle, and in channel catfish, the ovaries are paired, with each member of the pair of about equal size, located below the trunk kidney and swim bladder (Grizzle and Rogers 1976).

(ii) Known Biological Processes during Spawning – The processes associated with the time immediately before spawning in teleosts include the completion of oogenesis with the end of vitellogenesis, oocyte maturation, and ovulation. Initiation of oocyte maturation is indicated by the movement of the nucleus (germinal vesicle) to the periphery of the animal pole and micropyle, oocyte hydration, completion of the first meiotic division, initiation of the second meiotic division, and production of a secondary oocyte arrested at the second metaphase of meiosis for release and subsequent fertilization during spawning (Nagahama 1983). Previous studies on hormone-induced oocyte maturation of channel catfish have resulted in variable spawning rates, fertilization rates, and collection of non-viable eggs that were disintegrating or described as 'putrescent' (Lang and Tiersch 2007; Chatakondi et al. 2011).

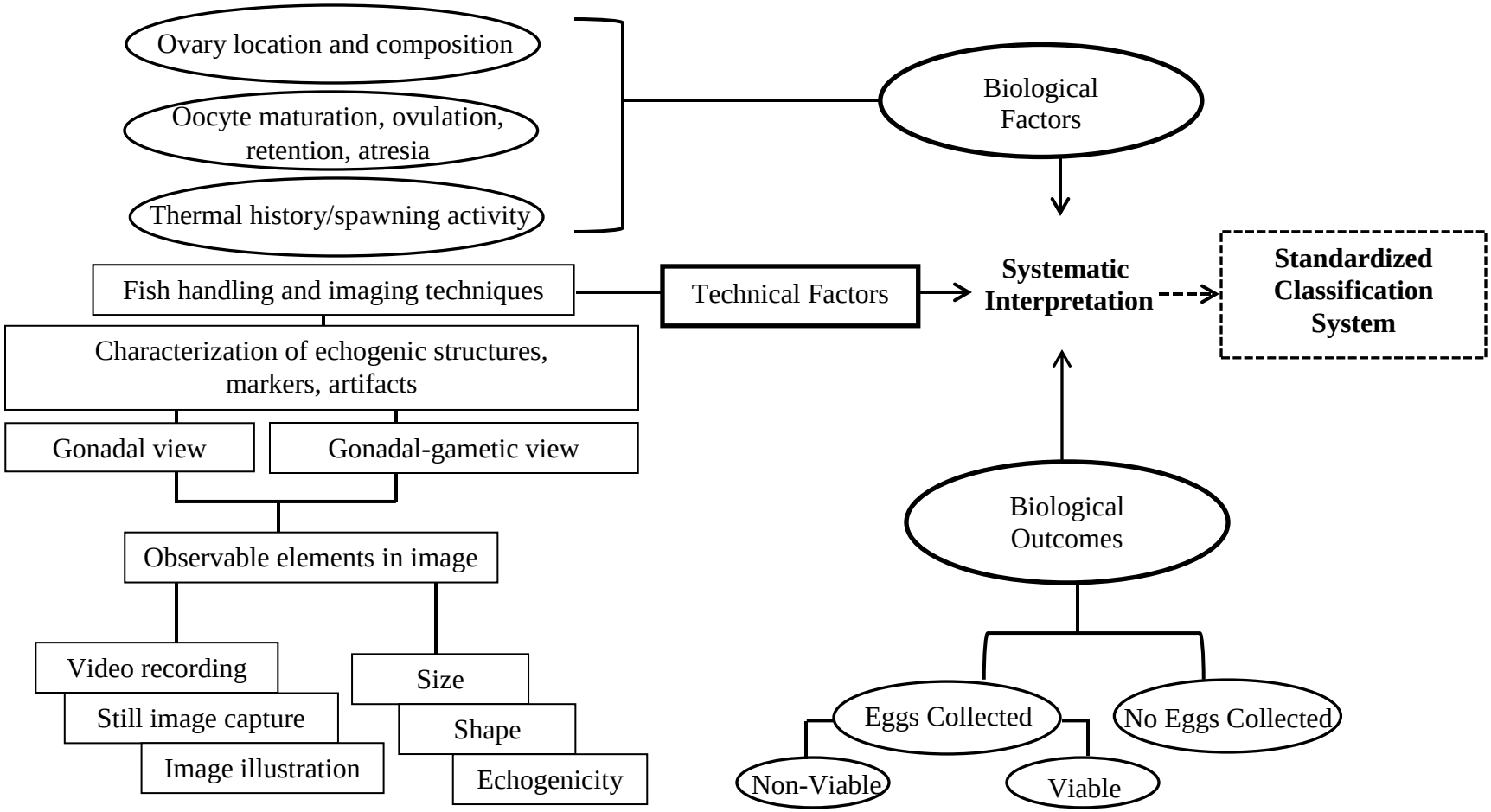


Figure 4.8 Biological (ovals) and ultrasonographic (rectangles) schematic components used for interpretation of images of channel catfish ovaries recorded during hormone-induced spawning. Biological insight was informed by biotic and abiotic factors, including the location and tissue components of the ovary, known gonadal processes occurring during artificial spawning (maturation, retention, atresia), water temperature, and observable biological outcomes. The integration of biological and ultrasonographic insights provided a standard and systematic interpretation approach for future development of a standardized classification system (i.e., dashed lines = do not exist at present, and should be the subject for future research). Ultrasonographic insight was informed by use of consistent fish handling and imaging techniques, including the use of the gonadal-gametic view and the size, shape and echogenicity of tissues.

(iii) Thermal History and Spawning – The ambient temperatures (19 - 21 C) at the start of heating for the first four spawning periods were close to the threshold temperature (21 C) used for calculating degree-days. Degree-day accumulation began 2 d after heating was initiated in January for the first two artificial spawning periods, and 1 day after heating was initiated in February for the third and fourth artificial spawning periods. Degree-day accumulation for fish held in ponds at ambient water temperatures in the last two spawning periods began in mid-March but the temperature profile was not consistently above 21 C until after mid-April. Images of ovaries and oocytes before injection were representative of reproductive condition of channel catfish 2 to 4 d after capture by seine. Although all fish during each spawning trial at the time of injection and ultrasound imaging shared the same thermal conditioning and degree-day profiles (Figure 4.1), the images recorded during monitoring of hormone-induced spawning were representative of varying biological processes.

(iv) Biological Outcome during the Spawning Trials – The biological outcomes observed were of fish from which fertilizable eggs were collected, of fish from which non-viable eggs were collected, and of fish which did not release eggs but instead retained their oocytes. The quality of eggs collected was evaluated based on neurulation data, and on external morphology of non-viable eggs. Eggs of high (> 50% neurulation) and low (< 50% neurulation) quality (Bates and Tiersch 1998) were collected. These eggs, especially cohorts of high quality eggs, were indicative of oocytes that underwent oocyte maturation, hydration, and ovulation. Two types of non-viable eggs were collected at the time of strip-spawning. Firstly, non-viable eggs were collected in clumps enmeshed in ovarian tissue. This clumped morphology strongly suggested these eggs were not fully ovulated. Secondly, non-viable eggs were collected that had lost

physical integrity and were visibly deformed, decomposing, or discolored (e.g. white, and pale). This disintegrating morphology indicated the process of atresia. Fish from which no eggs were collected indicated that the retained oocytes would presumably undergo mass atresia. The egg collection and neurulation profiles were evidence of ovarian and gametic development processes leading to oocyte maturation, collection of viable and non-viable eggs, or retention of oocytes and atresia.

Ultrasonography Insight. (i) Fish Handling and Imaging – The fish handling and imaging techniques used for viewing the ovary of unanesthetized, unrestrained, and submersed channel catfish provided the means to systematically view, record and decipher the orientation of the ovarian cross-section as it appeared in displayed images (Guitreau et al. 2012). Storing the appropriate settings in the 'pre-set' option of the ultrasound software ensured that any changes observed over time during monitoring of hormone-induced spawning would reflect changes in morphology of the anatomy and not changes caused by variation of methods or instrumentation within one group of settings.

(ii) Characterizing Echogenic Structures, Markers, Artifacts – Knowledge of the internal anatomy and location of organs in channel catfish, the positioning of the probe and fish, and an understanding of ultrasound-tissue interaction properties were the basis for identifying echogenic structures and anatomical markers during ultrasonography. The probe was positioned alongside the skin of the fish, with water as the ultrasound medium; therefore, the first structure to appear in the near-field view of the image was the skin of the fish, which was hyperechoic relative to the water in all the images recorded (Figures 4.2, 4.4 – 4.7). Secondly, the tissues alongside the

abdomen and under the skin appeared as a greyish, homogenous echogenic texture. The bone and airbladder were identified by positioning the probe with the cable end raised toward the top of the fish and anterior to the dorsal fin. The airbladder, located just ventral to the vertebrae between the head and trunk kidney, is filled with a mixture of gases including nitrogen, oxygen, and carbon dioxide (Grizzle and Rogers 1976; Lee 1981), and appeared bright white because bone (minerals) and gases do not conduct ultrasound (Toal 1996; Nyland et al. 2002). The gas content in the airbladder produced a reverberation artifact that disrupted ultrasonography of the ovary when the probe was positioned on the lateral side of the fish anterior to the dorsal fin (Figure 4.9).

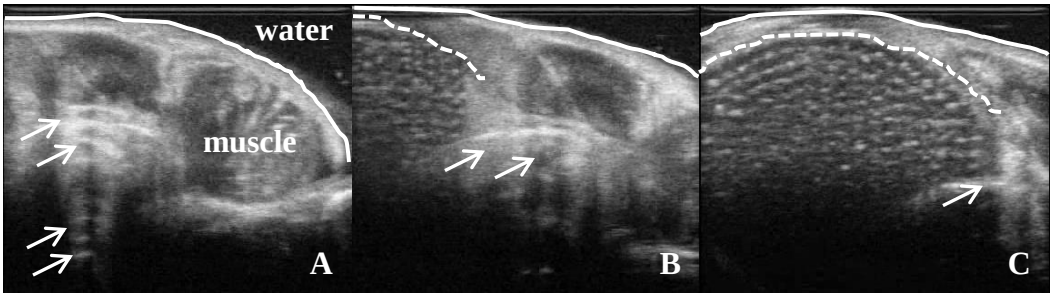


Figure 4.9 These still images were captured from the same video for illustration of ultrasound imaging artifacts (arrows) which did not properly represent internal anatomical structures. The airbladder produced hyperechoic reverberation artifacts which appeared as wide white parallel arches nearest the probe with weaker resonating echoes towards the far-field view. These images showed the progression of artifact appearance during image capture from prominent (A, B) to minimized or eliminated (C) so that the ovary was centered in the display image field of view. Water = ultrasound medium, white line = skin, muscle = muscle bundle, and dashed line = periphery of the ovary and oocyte cross-section.

To capture and record images with minimal interference from bone and gas, the spine and the airbladder were used for image orientation to locate the ovary, and the probe was then positioned away from these structures (Figures 4.3C, 4.5B, 4.6B). The ovary was identified by scanning the side of the abdomen, which encased presumably enlarged ovaries just before spawning (Brauhn and McCraren 1975; Markmann and Doroshov 1983). Finally, the gonadal-gametic view

displayed oocyte structures which were characteristic of lipid and fluid interactions with ultrasound. Lipid attenuates ultrasound (reducing the amplitude and intensity) and appears white to moderately white, and homogeneous fluids appear black or as black with white inclusions (complex cellular fluids) (Toal 1996). These easily visible anatomically constant structures – – skin, muscle tissue, spine, airbladder and ovary – – were used as reference points within the ultrasound images during real-time monitoring, and for recording BMP still images from AVI video images of catfish.

(iii) Observable Ultrasound Image Elements – The shape, size and echogenicity (the echo pattern, and ultrasound attenuation) of the ovary and oocytes were easily recognized and useful as key indicators for observations and interpretation of ovarian morphology and reproductive state during the spawning trials.

The Ovary – The ovarian cross-section appeared as oval in the gonadal and gonadal-gametic videos. The dorso-ventral axis of the fish was represented as the long axis of the ovarian cross-section oriented from the right to left direction in the image display (Figure 4.2). The lateral axis of the fish was represented as the short axis of the ovarian cross-section oriented from the top to bottom direction in the image display (Figure 4.2). The shorter axis was perpendicular to the skin where the probe was positioned and in the direction of ultrasound emission (Figure 4.2). From the gonadal view, the ovaries generally appeared as a uniform grey texture devoid of any internal structure, and the ovarian wall was difficult to view other than by observation of changes in echogenicity of adjacent structures (i.e. at the periphery of the ovary nearest to the probe, the adjacent muscle and skin) (Figures 4.4, 4.5). In the gonadal-gametic view, the location of the

ovarian wall was defined by the delineation of the oocytes encased within the oval shape of the ovary within the periphery of the ovarian cross-section, but the ovarian wall was thin and not resolved in the images (4.2B, 4.6, 4.7).

The Oocytes – The oocytes were clearly visible from the gonadal-gametic view. They appeared as multiple ovoid structures which filled the ovarian cross-section. Individual oocytes appeared as heterogeneous echoic structures; that is, the oocyte was composed of a white-to-light-grey appearance in the center (hyperechoic center) and graduated into a dark-ringed structure on the periphery (hypoechoic periphery) which demarcated a boundary zone. The overall size of the oocyte was defined by its heterogeneous structure (the hyperechoic and hypoechoic composition). Because the oocytes provided multiple interfaces for ultrasonography, the ovary appeared with a complex internal structure, with more resolution and clarity in the near-field view of the images, as the ultrasound signal was weakened (i.e. attenuated) during its transmission and interaction with ovarian tissues.

Ultrasound Image Interpretation. The collection of ultrasound image videos represented ovaries that shared the same thermal reproductive conditioning (Figure 4.1), but the readiness to spawn varied, as was indicated by the biological outcomes (egg collection, neurulation, atresia). These biological outcomes included females that underwent oocyte maturation including hydration and fertilization (Figures 4.4, 4.6, 4.10), females that retained their oocytes – neither released eggs in the observation tanks nor in manual strip-spawning attempts (Figures 4.5, 4.7, 4.11), and females from which two types of non-viable eggs were collected – clumpy eggs which

indicated the oocytes were not ready to be ovulated, and eggs which had lost their ovoid shape, and were physically disintegrating (Figure 4.12).

Ovarian images at the time of hormone injection of fish from which viable eggs were collected were characterized by distinct oocytes defined by a hyperechoic (brighter) center surrounded by a hypoechoic (darker) peripheral zone which together formed the ovoid shape of the oocyte. The oocytes were distinctly visible in the near-field view of the ovarian cross-section, but this clarity was lost towards the far-field view as ultrasound was attenuated (left panel, Figure 4.10). In comparison, at the time of egg collection the ovary and oocytes appeared enlarged and homogenous in some areas, and the oocyte peripheral zones were not distinct; these visible changes in size, shape, and echogenicity may be attributed to the intake of fluid during oocyte hydration (right panel, Figure 4.10).

Ovarian images of fish that retained their oocytes (no eggs collected) were characterized by similar image morphology at the time of injection compared to 67 to 80 h later (Figures 4.5, 4.7, 4.11). There was no noticeable change in the size, shape and echogenicity of the ovary and oocytes in these females during the spawning period (Figure 4.11) compared to images of females which underwent oocyte maturation (Figure 4.10). In the images of oocytes in Figure 4.10, a visual estimate (see vertical reference bar of the same size placed in the image at the time of injection and at the time of egg collection) indicated the distance between the skin and the ovarian periphery was reduced. However, in images of oocyte retention, the size of the ovary in the left panel at the time of injection remained similar to that in images recorded at 67 to 80 h (Figure 4.11).

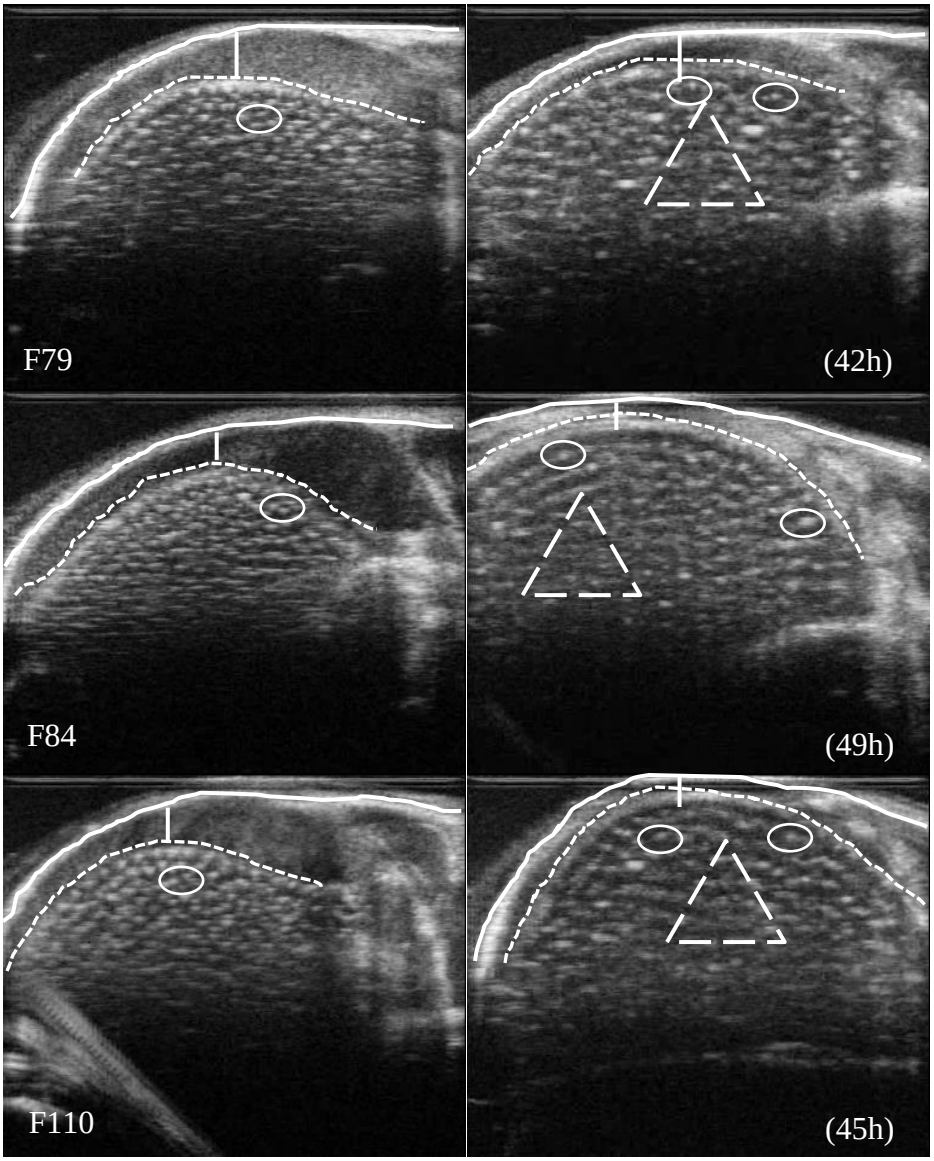


Figure 4.10 Images of oocyte maturation including hydration displayed a visually discernible change in size, shape, and echogenicity. Images of the same fish were recorded at the time of hormone injection (0 h) (left vertical panel) and at the time of egg collection (right vertical panel). Vertical bars of the same size were placed in between the skin (solid white line) and the periphery (dashed line) of the ovary as a visual reference – about 50% reduction of the reference bar in right panel images of enlarged ovaries. Enlarged oocytes were illustrated by same-sized oval white shapes, which encased 4 to 5 oocytes (left panel) and 1 oocyte (right panel). The echogenicity of the ovarian tissues on the left panel provided a global, organized oocyte view, with a light-grey appearance in the center which graduated into a dark-ringed structure in the periphery (oocyte boundary zone. Although hydrated ovary and oocytes retained the characteristic appearance of light-grey in the center and a darker periphery, oocyte boundary zones in some areas of the cross-section were not discernible as the predominant feature of the oocyte center appeared grey, enlarged, and lacked the defined dark-ringed structure of the oocyte periphery, thus imparting a homogenous appearance (triangle area) in the ovarian cross-section.

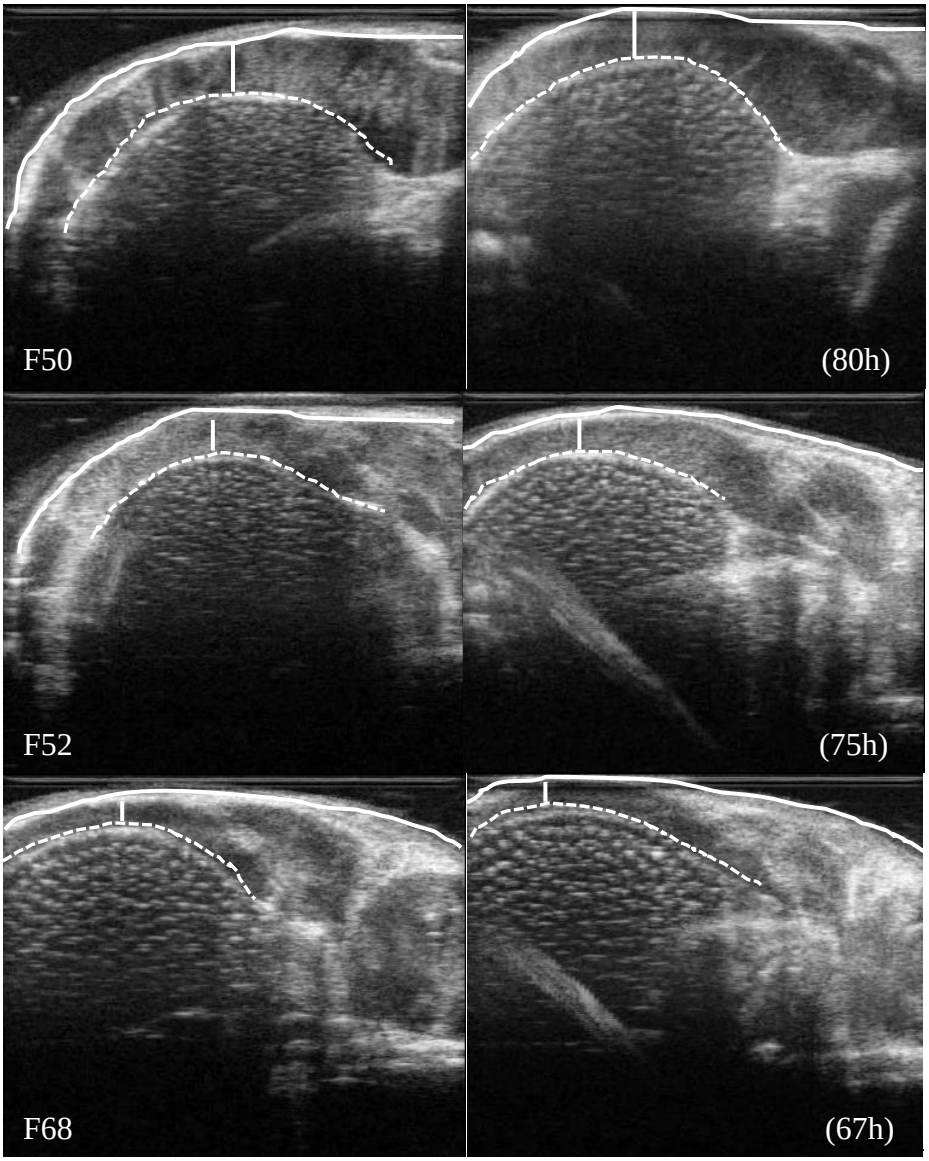


Figure 4.11 Representative images of ovaries from which no eggs were collected displayed minimal change in size, shape, and echogenicity of ovaries and oocytes. Images on the left vertical panel recorded at the time of hormone injection (0h) were of the same fish (indicated by the fish identification number) as images on the right vertical panel. Vertical bars of the same size were placed in the paired images of the same fish between the skin (solid white line) and the periphery (dashed line) as a visual reference of little or no change in the size of the ovary. The size, shape, and echogenicity of the oocytes in images on the left vertical panel remained similar to images of the right vertical panel – the oocytes appeared with the white-grey center graduating into a darker structure, but no drastic change in size, shape, or echogenicity was present as was distinctly observed for oocytes that underwent oocyte maturation illustrated in Figure 4.10.

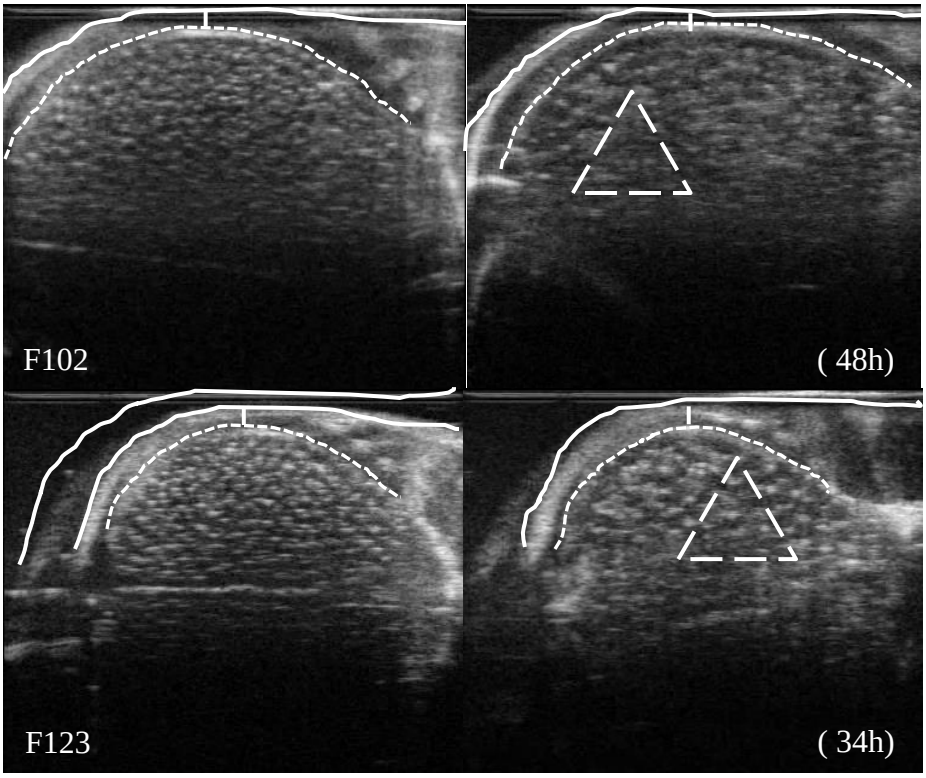


Figure 4.12 Representative images of ovaries from which non-viable eggs were observed upon collection as clumpy (F102) and deteriorating (F123) displayed enlarged ovaries of similar size and shape at the time of injection (0 h) and egg collection. However, the shape and echogenicity of the oocytes changed drastically and consequently the echogenic pattern of the ovary changed. Images on the left vertical panel recorded at the time of hormone injection (0 h) were of the same fish (indicated by the fish identification number) as images on the right vertical panel recorded before egg collection. Vertical bars of the same size were placed in the paired images of the same fish between the skin (solid white line) and the periphery (dashed line) of the ovarian cross-section as a visual reference for little or no change in the size of the ovary. However, the shape and echogenicity of the ovary, and in particular, the oocytes, changed. Although oocyte boundaries were visible before egg collection, oocytes appeared irregular in shape, with no distinct center (illustrated by the triangle area) in the majority of the ovary cross-section.

Oocytes did not appear hydrated (they were not expanded), and they remained with a heterogeneous echogenicity including visible shapes and boundaries at the time of injection and at the end of the spawning trial (Figure 4.11).

Ovarian images of fish which produced the two types of non-viable eggs displayed ovaries that appeared large and closer to the skin (Figure 4.12). Visual observation of the distance between

the skin and the periphery of the ovary indicated the ovarian size and shape appeared the same before injection and at egg collection (Figure 4.12). However, the size and shape of the oocytes changed from an organized and discernible appearance to irregular shapes and sizes with a diffuse boundary and highly irregular appearance within the ovary (Figure 4.12). Rather than with no identifiable oocyte boundary zone, and where some oocyte boundary zones were visible (prior to egg collection), the size of the oocytes was difficult to visually estimate because of their deformed appearance (Figure 4.12). This oocyte morphology in turn changed the echogenic texture of the ovarian cross-section to a predominance of homogeneous light or dark-grey areas within the ovary.

Discussion

Hormone-Induced Spawning and Fertilization. Spawning Latency, Spawning Rate, Neurulation Rate. Artificial spawning of channel catfish in this study was done in the manner of previous studies in a recirculating system equipped with a plexiglass viewing window for observation of spawning behavior of male-female and female-female pairings (Bates and Tiersch 1998; Pawiroredjo 2004), and for thermal conditioning of fish before and during the natural spawning season in mixed-sex (male and female) and same-sex (all-male, or all-female) ponds (Lang et al. 2003; Lang and Tiersch 2007). No difference was observed in the proportion of females that spawned (i.e. produced eggs) in male-female pairings over a 3-year period, and between male-female pairs and all-female groups (Bates and Tiersch 1998). The latency period varied significantly over the 3-year period for the group of fish spawned in 1996, but it did not vary between paired and group females within the same year (1996). Fertilization rates varied significantly over the 3-year period for the group of fish spawned in 1995, and it was significantly higher for paired females compared to grouped females (Bates and Tiersch 1998).

So, this variation was attributed to variability in the reproductive readiness of females, to the differential response to hormones, and to the phase of the natural spawning season in southern Louisiana (Aquaculture Research Station, Baton Rouge, Louisiana) divided into early, middle, and late phases during which females were hormone-induced to spawn. In particular, higher egg quality (> 50% neurulation) was reported for a 4-week period, from early May to early June, and lower egg quality (< 50% neurulation) were reported for eggs from females held for longer periods of time at spawning temperatures (Bates and Tiersch 1998).

The use of degree days as a standard unit and guideline to describe and quantify heating requirements provided reference points that could be used to compare results from future and past data about channel catfish spawning (Pawiroredjo et al. 2008). This study used 21 C as the threshold temperature for degree-day calculation, the threshold recommended for predictive and comparative functions (Pawiroredjo et al. 2008). The degree-day values and spawning probability guidelines corresponding to channel catfish spawning in 0.04-ha ponds in southern Louisiana were reported as 57 – 81 degree-days for the onset of spawning (10% of fish), 99 – 129 degree days for the median of spawning (50% of broodstock), and 150 – 172 degree days for the conclusion of spawning (90% of broodstock). The degree-day values calculated in this study (Figure 4.1), when compared to the onset, median and conclusion of spawning degree-day guidelines, showed that all the spawning trials, except for one, were within or exceeded the degree-day range for conclusion of spawning (150 – 172 degree days). The value (116 degree days) for the first trial was the only degree day value in this study that fell within the range for the median of spawning (99 – 124 degree days), and corresponded to the highest spawning rate of the six spawning trials (Figure 4.2). The prolonged exposure to spawning temperatures may

be a major contributing factor explaining the low rate of spawning (females producing viable eggs/females injected) in this study (Figure 4.2). This concurs with the suggestion that the longer females are held at spawning temperatures the lower the egg quality (Bates and Tiersch 1998).

Use of "Gonadal" or "Gonadal-gametic" Viewing Areas. Fish Handling, Imaging, and Settings. Monitoring of channel catfish during artificial spawning was an intensive process, with observation of spawning progress potentially necessary every 2 hr, and ultrasound imaging and recording at the time of injection, at 18 to 20 hr after injection, and every 2 to 4 hr for the duration of the spawning trails. The movement of fish was evident in video recordings because of the free-swimming and unrestrained handling of the fish in the spawning tanks. This lack of physical handling was intentional in order to cause the least stress to the fish as possible. Videos recorded using this completely unrestrained method showed caudal musculature and fins before the ovaries came into the display image as the ultrasonographer aligned the probe alongside the dorsal fin. One main recommendation arising from this was to lightly but firmly hold the fish by the caudal peduncle to quickly align the fish and probe for effective positioning and imaging.

Whereas the initial development of fish handling and imaging techniques minimized handling and provided consistent procedures for ultrasonography of channel catfish ovaries (Guitreau et al. 2012), this study evaluated the utility of "wide-angle" and "close-up" viewing areas for monitoring ovarian reproductive condition during spawning trials. The emission of either high (8 MHz) or low (5 MHz) frequencies in combination with the other control settings used (Table 3) generated distinct vantage points with different echogenic structures, patterns, and resolutions.

For instance, the lower frequency (5 MHz) had a lower axial resolution, and in conjunction with raising the dynamic range (set at 61 dB, which lowered the image contrast) and other settings used provided a broad view of the gonad, but not of individual oocytes. The gonadal view, therefore, was generated by using a combination of ultrasound control settings that allowed for deeper penetration and wider cross-section imaging, but low resolution (Figures 4.2A, 4.4, and 4.5). This type of image was useful for capturing a real-time quick view of the paired ovaries, but it lacked the resolution necessary for monitoring oocytes.

In contrast, the ability to discriminate between two points along the axis of the ultrasound beam (i.e. the axial resolution) which is superior in higher frequencies (e.g. 7.5 MHz as a higher frequency, vs 3.5 MHz as a lower frequency) (Nyland et al. 2002), in conjunction with lowering of the dynamic range control (set at 56 dB, which raised the image contrast) and other basic settings such as the gain and power (Table 4.3) provided a focused view of an ovary encasing distinct oocytes (Figure 4.6, 4.7).

The gonadal-gametic view was generated using a combination of settings that allowed for higher resolution, less ultrasound penetration, and a clear view of the ovary and oocytes closest to the probe (Figure 4.2B, 4.6, 4.7). The hyperechoic center of the oocytes can be attributed to the echogenicity or brightness caused by lipoproteins in the yolk globules that concentrate centripetally forming a mass of yolk fluid (Tyler and Sumpter 1996) as the oocyte prepares for maturation and ovulation. The peripheral hypoechoic zone of the oocytes (the dark ring-like structure) may be indicative of a zone of fluid absorption caused by the cytoplasm of the oocyte which lacked yolk, ovarian fluids within the ovary and between oocytes, or a combination of

these fluids. Ultrasound interaction with pure fluids appears black, but fluids such as blood which contain small particles cause weak echoes (Lutz 2011). This type of image was useful for a close-up, explicit view for observation of the reproductive condition of the gonad and oocytes during hormone-induced spawning.

Capturing and Formatting BMP Images – The formatting of ultrasound images reported in this study provided the steps used to illustrate the appearance of ultrasound images of the channel catfish ovary and oocytes. Descriptions of ultrasound image formatting for illustrative purposes builds familiarity with how images appeared in the BMP images captured from AVI images. This provided a context for understanding the presentation of figure illustrations, which may be a useful frame of reference for standardized reporting of imaging processing. Reporting this methodology would contribute to increased ease of understanding for readers and those unfamiliar with the technology. Therefore, the approach proposed for incorporation of this imaging technology in research and field applications is to break ground by explaining procedures as simple and clearly as possible. This means that, in addition to the recommendation for text descriptions (with specific references to external anatomy) and pictorial illustrations (pictures or sketches) for reporting the positioning of fish with respect to the probe and the image as it appears on the screen (Novelo and Tiersch 2012), steps should be provided for how the ultrasound image was generated, recorded (i.e. video or still image) and processed for use in ultrasonography illustrations and interpretation.

Development of a Standardized and Systematic Approach for Ultrasound Image Interpretation. Biology and Ultrasonography. This approach was based on the concept of

incorporating the different procedures and processes involved in biological and ultrasonographic systems. Interpretation of ultrasound images of the channel catfish ovaries was built on background knowledge of the biological and environmental influences on reproductive condition of the ovary, which was utilized as a precursor to the physical process of ultrasonography during which the biological insight from background knowledge and observed biological outcomes were used for defining and interpreting images (Figure 4.8). Key features within the image generated by the gonadal-gametic view were identified for their utility in denoting changes in the ovarian echogenic morphology. These key features were the basis for discriminating ovarian and oocyte morphology of fish that underwent oocyte maturation (Figure 4.10), of fish that retained their oocytes at 70 to 80 h after hormone injection (Figure 4.11), and of fish that produced non-viable eggs (Figure 4.12). This method for interpretation of images provided the necessary framework for development of a standardized classification system for ultrasound images of ovaries before and after hormone-injection.

Summary and Conclusions

In this study, use of ultrasonography provided new and real-time imaging data on ovarian morphology during monitoring of the biological processes occurring during hormone-induced spawning of channel catfish. The gonadal-gametic (close-up) view yielded imaging morphology for ovaries and oocytes which was useful for deciphering the reproductive state of the fish. To examine the robustness of this approach, fish were observed before and during the natural spawning season by standardization of thermal conditioning. Multiple factors influencing the interpretation of images of channel catfish ovaries and oocytes were placed within a systematic framework for defining the ultrasound image morphology of biological processes occurring during hormone-induced spawning. In particular, the size, shape, and echogenicity of ovarian

morphology were defined for unovulated and atretic oocytes, and for oocyte growth during the oocyte maturation phase, including hydration. Future studies should address approaches for standardized classification of catfish ovarian reproductive condition for use in research and commercial hatcheries.

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Chapter 5
Development, Use, and Evaluation of a Preliminary Ultrasound Imaging Assessment Index for Channel Catfish Ovarian Development

The channel catfish *Ictalurus punctatus* is the most economically important cultured food fish species in the United States of America (USA), with total sales in excess of US $450 million per year from 1998 to 2008 (USDA 2000-2010). However, its production has recently declined, with total sales of domestic catfish products valued at US $341 and $342 million in 2012 and 2013 (Hanson and Sites 2014). The method of channel catfish commercial seedstock production until 2001 was pond-based reproduction, which relied on the natural mating of males and females in spawning containers (Hargreaves and Tucker 2004). However, in 2001 US catfish hatcheries started commercial production of F_1 hybrid seedstock obtained from crossing of the female channel catfish and the male blue catfish *Ictalurus furcatus* (Chatakondi 2012). This production increased from an initial 2 million fry in 2001 to about 111 million in 2011, which was equivalent to 15% of 732 million catfish fry produced in 2011 (Chatakondi 2012).

Commercial use of artificial spawning to produce hybrid seedstock introduced new hatchery practices which required more specialized labor and knowledge than pond reproduction (Avery et al. 2005; Phelps et al. 2011; Novelo and Tiersch 2012b). One critical component is the selection of the females for viable egg production (Avery et al. 2005; Novelo and Tiersch 2012b). The method commonly used for assessing ovarian development in the commercial hatchery is based on expectations associated with the external morphology of the fish and ovarian development. If females display the 'correct' or 'desirable' morphological features such as distension of the abdomen and a reddish swollen papilla, the expectation is that they will likely produce viable eggs during artificial spawning (Lee 1991). However, the criteria for

deciding whether or not to inject a fish for artificial spawning are subjective and vary based on the experience of the assessor and amongst different workers at different farms (Phelps et al. 2011).

There are several research methods for selecting females for induced spawning, such as catherization and visualization of the germinal vesicle, or direct viewing of the oocytes through endoscopy. The utility of ultrasound imaging for assessing commercially relevant numbers of fish has been reported for Atlantic cod *Gadus morhua* (~ 2,000 fish in two studies), for the anadromous form of rainbow trout *Oncorhynchus mykiss* (> 1,000 in one study), and for red hind *Ephinephelus guttatus* (> 800 fish in one study) (Novelo and Tiersch 2012a). Its potential for viewing and assessing the reproductive state of the ovary with commercially relevant, rapid, and consistent handling and imaging procedures was previously reported for channel catfish (Guitreau et al. 2012). The goal of this study was to develop and evaluate a preliminary ultrasound imaging classification approach for ovarian reproductive assessment during three years of work to generate biological insight leading to the development of a well-defined classification index for use as a decision-making tool with direct relevance to commercial hatcheries. The objectives were to: (1) develop an ultrasound imaging reproductive classification index for assessment of channel catfish ovarian development during all phases of the ovarian cycle, (2) to evaluate fertilization of eggs collected in 10 spawning trials during 2008 to 2010, and (3) evaluate the accuracy of the expected and observed outcomes during artificial spawning of the ultrasound imaging assessments for the categories developed. This study characterized and evaluated ultrasound categories for assessing channel catfish ovarian development and analyzed the rater success in categorizing images of ovarian development.

This characterization was based on the predicted (expected outcome) and observed outcomes of egg collection and fertilization proposed for each classification used in the reproductive assessment of channel catfish.

Methods

Development of the Ultrasound Imaging Classification Index. Equipment and Settings. The ultrasound equipment used was a portable ultrasound unit (Classic TelaVet 1000™ Veterinary Digital Ultrasound Module, Telemed UAB, Vilnius, Lithuania), and a linear probe (model LV7.5/60/96). The ultrasound controls were real-time B-Mode, with a probe frequency set at 8 MHz (in 2008) or at 5 and 8 MHz (in 2009 and 2010), the 'Transmit' (mm) control (focusing at a depth location during transmission) set at 10, scanning depth at 80, 'dynamic range' (dB) (controlling image contrast) was set at 56 or 62, power (acoustic control of ultrasound beam) was set at 90 or 100%, 'overall gain' (uniform amplification of returning echoes, which controls voltage amplitudes and echo brightness) was set at 70 (2008 – 2009) or at 40, 70, 76 and 82 (2010), 'zoom' (the zoom ratio) at 100%, 'frame average' (setting how many frames will be averaged to lower background sound artifacts) was set at 4 (2008 – 2009) or 0 (2010), the 'reject' control (alters the threshold for stronger or weaker range of returning echoes, ultrasonic signal rejection to reduce noise visibility) was set at 0, and time-gain compensation (TGC) controls (horizontal slider controls that adjust the gain at specific depths) were adjusted to 0 (TGC control 1), 20 (TGC control 2), 40 (TGC control, 60 (TGC control 4), and 80 mm (TGC control 5). After ultrasound images of ovaries were assessed for reproductive development, the images were recorded in the following formats: Audio Video Interleave (AVI), ultrasound image format (USI), Windows Bitmap Image format (BMP), and Tagged Image Format Files (TIFF).

Ultrasound Imaging of Ovarian Recrudescence. In Louisiana, the recrudescence phase and initiation of vitellogenesis of the channel catfish ovarian cycle were associated with a slow growth period of oocytes and ovary during October and November leading into a period of rapid oocyte growth from March to April preceding the spawning phase (Trant et al. 1997; Banks et al. 1999). Ultrasound images were recorded during six sampling dates within the recrudescence phase of the ovarian cycle of adult (3 - 5 yr) channel catfish broodstock (obtained from Baxter Lands Company, Dumas Arkansas) conditioned for reproduction at the Louisiana State University Agricultural Center-Aquaculture Research Station (LSUAC-ARS), Baton Rouge, Louisiana (Table 5.1). Earthen ponds (0.16 – 0.30 ha) were at ambient temperatures, and the broodstock sampled ranged from 2 to 2.6 kg (mean weight) and from 61 to 64 cm (mean total length).

Table 5.1 The sampling date, number of fish, weight and total length (average ± SD), broodstock pond sampled (Pond ID) and the size (ha) were listed for ultrasound imaging of channel catfish ovaries in the recrudescence period during 2008 to 2010 at the LSUAC-ARS in Baton Rouge, Louisiana. Ultrasound images were recorded for fish collected during the seven sampling dates in Windows Bitmap Image format (BMP), and Tagged Image Format Files (TIFF).

Sampling	Number	Image	Weight	Length	Pond	
date	of fish	format	(kg)	(cm)	ID	Size (ha)
10/28/2008	108	BMP	2.4 ± 0.4	-	B6	0.30
11/05/2008	92	BMP	2.6 ± 0.4	64 ± 3	B4	0.30
11/14/2008	46	BMP	2.2 ± 0.7	61 ± 5	M7	0.16
02/03/2009	253	BMP	2.5 ± 0.5	63 ± 3	H4	0.16
03/13/2009	141	BMP	2.2 ± 0.4	61 ± 3	H7	0.16
03/25/2010	65	TIFF	2.0 ± 1.3	61 ± 3	B6	0.30

Ultrasound Image Sampling During the Spawning Period. Ultrasound images of ovaries were recorded for fish held for reproductive conditioning at ambient temperatures in 12 ponds (0.04 – 0.30 ha) during the natural spawning phase, and for fish conditioned to spawn in 4 ponds (0.04 ha) using established geothermal control protocols of raising the temperature by 2 C/day

from the ambient temperature until 28 C was reached (Hall et al. 2002; Lang et al. 2003; Lang and Tiersch 2007) (Table 5.2). Spawning cans were removed and a date was selected for fish collection by seine for ultrasound imaging and artificial spawning after 10 to 20% of the females had spawned, as observed with the collection of egg masses (Table 5.2).

Table 5.2 Stocking, thermal conditioning, egg mass collection and fish capture by seine for 10 spawning trails during 2008 to 2010. PI = pond identification code; PS = pond size, ha of water; F = Females stocked; M = Males stocked; Date = date of stocking; T = temperature; H = use of geothermal water for heating ponds for reproductive conditioning before the natural spawning season; A = use of ambient temperatures for reproductive conditioning and spawning during the natural spawning season; Egg Masses = egg masses collected from spawning cans.

Trial	Stocked							
ID	PI	PS	F	M	Date	T	Egg Masses	Fish Capture
I	G2	0.04	30	10	02/22/2008	H	6	04/04/2008
II	G6	0.04	30	10	02/29/2008	A	3	05/26/2008
III	G7	0.04	27	9	05/13/2008	A	4	06/02/2008
IV	R2	0.02	27	9	02/03/2009	A	6	04/25/2009
IV	R3	0.02	27	9	02/03/2009	A	8	04/25/2009
V	G2	0.04	27	9	03/13/2009	H	2	05/09/2009
V	G3	0.04	27	9	03/13/2009	A	4	05/09/2009
VI	B3B	0.30	170	-	11/14/2009	A	-	05/26/2009
VII	H4	0.16	200	-	11/05/2009	A	-	06/01/2009
VII	H7	0.16	310	-	10/28/2009	A	-	06/01/2009
VIII	G1	0.04	30	10	02/11/2010	H	2	03/30/2010
VIII	G2	0.04	30	10	02/11/2010	H	4	03/30/2010
IX	B9	0.30	-	-	-	A	-	06/05/2010
X	G1	0.04	39	10	05/07/2010	A	2	05/17/2010
X	R2	0.02	30	10	03/25/2010	A	3	05/17/2010
X	R3	0.02	30	10	03/25/2010	A	3	05/18/2010

Water temperature of the ponds was recorded in 0.5- to 1.0-h intervals with data loggers (SK100 Dickenson Temperature data loggers; Addisson, IL, USA). The temperature data collected for each pond were used to generate degree-day thermal profiles by calculating the sum of the daily difference between the mean daily temperature and 21 C, the recommended threshold temperature from the date of stocking to the date of fish capture by seine for ultrasound imaging prior to artificial spawning (Pawiroredjo et al. 2008). These degree-day profiles were compared

to the degree-day guidelines for the onset (57 – 81 degree-days, 10% spawning), middle (99 – 129 degree-days, 50% spawning) and conclusion (150 – 172 degree-days, 90% spawning) of spawning developed using channel catfish broodstock at the LSUAC-ARS (Pawiroredjo et al. 2008).

Fish Handling and Ultrasonography Procedures. The fish captured by seine from broodstock holding ponds during the recrudescence period (Table 5.1) were placed into a 2,800-L fish hauler provided with compressed oxygen and were transported to concrete 1,900-L flow-through raceways into which running water from an ambient temperature pond was pumped. Each raceway held 50 to 60 fish. The fish captured by seine from broodstock ponds during the ten spawning trails (Table 5.2) were placed in a 1,200-L fish hauler provided with compressed oxygen, which was stationed near the wet laboratory and used as a temporary holding unit during ultrasonography of the ovaries.

After the fish were in temporary holding systems (raceways or fish hauler), they were captured and moved using dip nets and fish baskets one at a time for weight and length measurements, then moved into a portable 49-L cooler (Sportsman™ 52 Quart, Igloo Products Corp., Katy, TX, USA) which was half filled with water (20 - 25 L, enough to submerge the fish). After the fish were in the cooler and submerged, the ultrasonographer positioned the fish by the caudal peduncle in an upright swimming position and first placed the probe (ultrasound transducer) alongside the dorsal fin near (0 - 1 cm) the skin. Ovarian morphological development was viewed and assessed by moving the probe alongside the lateral aspect of the fish towards the anterior and the posterior of the ovary (as displayed in the monitor) in the area between the

pectoral and the pelvic fin. This provided a general scan to quickly obtain a view of the entire ovary and a general assessment of ovarian development. Finally, the probe was held in position alongside the dorsal fin and once the image of the ovarian cross-section was centered and the skin surface of the fish was within 0 to 1 cm of the probe in the ultrasound image display, images were recorded (Tables 5.1 and 5.3). The fish was immediately removed from the cooler after which the next fish underwent the same procedure.

The development of the ultrasound imaging classification index for evaluating adult channel catfish ovarian development was based on: (1) the use of the initial fish handling and imaging procedures developed (Guitreau et al. 2012); (2) interpretation of ultrasound images based on a standardized and systematic approach to assessing the reproductive state of the ovary (Chapter 4, this dissertation); (3) the definition of ultrasound imaging categories of ovarian development based on the visibility, relative size and appearance of the ovary and oocytes coinciding with the recrudescence, spawning and regression phases of the ovarian cycle, and (4) development of a preliminary ultrasonography pictorial template representative of the ultrasound imaging categories used to assess ovarian development. Images recorded in each assessment category during the sampling dates (Tables 5.1 and 5.2) were assigned random numbers using the random excel function. The random numbers generated were sorted from the smallest to highest value. The first two images were selected for figure illustrations as representative images of the assessments.

Hormone-Induced Spawning and Fertilization. Hormone Administration and Egg Collection. The procedures for hormone injection and monitoring for ovulation and release of

eggs followed the guidelines in the hybrid catfish production manual (Avery et al. 2005). Female channel catfish selected for artificial spawning in Trial I were injected with a priming dose of 2 mg of common carp pituitary extract (CPE) (Stoller Fisheries, Spirit Lake, IA, USA) on the date of fish collection with a resolving dose of 8 mg of CPE/kg fish 16 h after the first injection. Fish selected for hormone artificial spawning in Trials II – X were injected with a priming dose of 20 µg of luteinizing hormone-releasing hormone analog (LHRHa) on the date of fish collection and ultrasound imaging assessment, and a resolving dose of 80 µg of LHRHa/kg of fish 16 to 18 h apart. Fish were examined for egg release and readiness for manual egg collection by applying gentle pressure on the abdomen 20 to 30 h after hormone injection and every 2 h thereafter. If eggs were readily released, fish were anesthetized with 150 to 200 ppm of tricaine methanesulfonate (Western Chemical Inc., Ferndale, WA, USA) for egg collection (Avery et al. 2005; Lang and Tiersch 2007) and eggs were stripped directly into greased food-grade plastic bowls containing modified Hanks' balanced salt solution (HBSS) which was adjusted to ~ 295 mOsm/kg measured by a vapor pressure osmometer (Wescor model 5520, Wescor Inc., Logan, UT, USA) (Tiersch et al. 1994; Christensen and Tiersch 1996). In total, 43 fish were injected with CPE or LHRHa in 2008, 88 fish in 2009 and 79 fish in 2010 (Table 5.3).

Egg Count and Fertilization – After collection, the eggs were rinsed with HBSS to remove any blood, and the total egg volume was recorded; if any clumps were present, they were removed with metal tweezers and the volume of eggs was recorded a second time. To estimate the number of eggs collected per female, the egg counts of two or three 5-mL egg volumes were recorded and the average eggs/mL calculated was multiplied by the total egg volume collected for each fish after removal of any clumps.

Table 5.3 Data on fish captured by seine for ovarian ultrasound imaging assessment before ten artificial spawning trails. PI = pond identification code; FC = females collected by seine; FI = The number of females injected with Carp Pituitary Extract in Trial I or Luteinizing Hormone Releasing Hormone analogue in Trials II to X; L = total length (cm) average ± SD deviation; W = weight (Kg) average ± SD. Ultrasound images were recorded for fish collected during the ten spawning trials in the following formats: Audio Video Interleave (AVI), ultrasound image format (USI), Windows Bitmap Image format (BMP), and Tagged Image Format Files (TIFF).

Trial	Artificial Spawning						
ID	PI	FC	Image Format	FI	L (cm)	W (Kg)	Dates
I	G2	39	AVI	22	59 ± 4	2.1 ± 0.4	April 4-7, 2008
II	G6	15	USI, BMP	7	-	1.9 ± 0.2	May 26-30, 2008
III	G7	27	BMP	14	-	1.8 ± 0.5	June 2-6, 2008
IV	R2	22	AVI, USI	14	-	2.5 ± 0.4	April 25-29, 2009
IV	R3	22	AVI, USI	13	-	2.5 ± 0.4	April 25-29, 2009
V	G2	22	AVI, USI	2	-	-	May 9-14, 2009
V	G3	23	AVI, USI	8	-	-	May 9-14, 2009
VI	B3B	121	AVI, USI	33	61 ± 6	2.2 ± 0.5	May 26-30, 2009
VII	H4	-	-	-	-	-	June 1-6, 2009
VII	H7	67	AVI, USI	18	-	2.5 ± 0.4	June 1-6, 2009
VIII	G1	30	TIFF	13	-	2.3 ± 0.4	March 30-April 3, 2010
VIII	G2	23	TIFF	11	-	2.3 ± 0.4	March 30-April 3, 2010
IX	B9	75	TIFF	21	60 ± 5	2.3 ± 0.5	May 5-10, 2010
X	G1	28	TIFF	8	62 ± 5	2.6 ± 0.4	May 17-22, 2010
X	R2	32	TIFF	8	61 ± 4	2.3 ± 0.4	May 17-22, 2010
X	R3	30	TIFF	18	61 ± 3	2.2 ± 0.3	May 17-22, 2010

Monolayers of eggs at the bottom of 100-mL tri-corner plastic beakers (Thermo Fisher Scientific Inc., Suwanne, GA, USA) were used as fertilization units. To estimate the number of eggs in one monolayer at the bottom of the beakers, three monolayer egg samples were poured and the average egg count was calculated for each female. Monolayers of eggs were poured into nine beakers for each female, 0.5 mL of sperm were added to each monolayer from three males (three replicates per male for each female), gametes were activated with 10 mL of hatchery water and after 5 min 10 mL of hatchery water was added for 10 min, after which the eggs were incubated in a recirculating hatching system. Eggs undergoing embryo development at 30 to 72 h after gamete activation were counted for each of the nine monolayers per female. The fertilization

estimate of each female was calculated as the ratio of the average count of eggs undergoing embryo development divided by the average count of eggs in the female monolayer sample.

Statistical Analysis – The main focus of this study was the development of an ultrasound imaging classification index to represent the ovarian cycle, and for this to be used as a tool to decide whether or not to inject fish for artificial spawning to increase spawning efficiency. Each spawning trial was performed on different dates and presented a full range of variation in ovarian development suitable for testing the utility of ultrasound imaging. For instance, testing the use of different hormones in the statistical models was not attempted because of the imbalance in assigning hormone treatments. Only 1 trial received one hormone (CPE), and the remaining 9 treatments received another (LHRHa). In addition, previous studies focusing on the use of heated ponds and ambient reproductive conditioning, and the use of channel catfish and blue catfish males, have indicated these had no effect on the fertilization of eggs (Lang et al. 2003, Lang and Tiersch 2007). Because all fish were combined into a single response variable in this study, no attempt was made to compare hormones because of a lack of replication, and the trial effect represented differences in all conditions as single variable. Therefore, the generalized linear mixed model (GLIMMIX) procedure of the Statistical Analysis Software (SAS) system version 9.3 for Microsoft® Windows® (SAS Institute Inc., 2012, Cary, NC, USA) was used to test for differences caused by the spawning trial (n = 10 trials) on fertilization (Statistical Analysis 5.1, Appendix B). The statistical model included the spawning trial as a fixed effect, and the combination of logit link and binomial distribution was used in the model.

Accuracy of the Ultrasound Imaging Classification Assessments. The accuracy of the ultrasound imaging assessments was evaluated based on the expected (i.e. hypothesized) and observed outcomes during hormone-induced spawning trials for each category. Each category was associated with one of two expected egg collection outcomes during hormone-induced spawning. Females were expected to: (1) produce viable eggs, or (2) to not produce viable eggs based on the categorical assessment. Females were considered to produce viable eggs if the fertilization rate estimated was of low (<50%) or high quality (≥50%) (Bates and Tiersch 1998). Females were considered to not produce viable eggs if (i) no eggs were collected; (ii) eggs were collected but were physically disintegrating; (iii) eggs were collected but were assessed as 0% fertilization estimate (i.e. no embryo development or physical degradation of eggs after 30 – 72 h of sperm and egg activation). The raw data of the expected and observed outcomes of fish injected in each category were tabulated for 10 spawning trials. The percent agreement of the observed and expected outcomes (Total Observed/Total Injected x 100) was calculated for each ultrasound imaging categorical assessment of channel catfish ovarian development.

Statistical Analysis. The PROC GLIMMIX procedure of SAS was used to test for differences in expected and observed outcomes of the ultrasound imaging categories used (Statistical Analysis 5.2, Appendix B). The expected outcome, year, and the interaction of expected outcome and year were included as fixed effects in the model and the observed outcome as the response variable. An error matrix (a cross-tabulation table) of the actual and predicted response was generated based on the actual biological outcome during artificial spawning and the predicted outcome (i.e. expected outcome) from the previous analysis to assess the accuracy (likeliness of

a correct assessment) of the ultrasound image classifications used in assessments of ovarian development. Differences in treatment means were considered significant at $P < 0.05$ for all statistical tests.

Results

Development of the Ultrasound Imaging Classification Index. A total of 915 ovarian ultrasound images were recorded during October through June in 2008 to 2010. One ultrasound image was recorded for each catfish at the time of fish capture by seine (Tables 5.1 – 5.4). The number of images recorded in the recrudescence phase of the ovarian cycle was 705, with one ultrasound image recorded for each fish (Table 5.4). The average time required for the image capture ranged from 32 s to 75 s per fish (Table 5.4). The remaining 210 images were recorded of fish collected by seine during the 10 spawning trials (Table 5.3).

Table 5.4 The sampling date, number images recorded, and the time (average ± SD) between consecutive images recorded were listed for ultrasound imaging of channel catfish ovaries in the recrudescence period during 2008 to 2010 at the LSUAC-ARS, Baton Rouge, Louisiana.

Sampling date	Number of images recorded	Time between images recording
10/28/2008	108	47 s ± 54 s
11/05/2008	92	32 s ± 28 s
11/14/2008	46	35 s ± 15 s
02/03/2009	253	54 s ± 68 s
03/13/2009	141	55 s ± 55 s
03/25/2010	65	75 s ± 44 s

The ultrasound images captured the range of a continuous ovarian development spectrum during the sampling dates (Figures 5.1 – 5.11). This continuum was represented by the development of an ultrasound imaging reproductive index comprised of 7 categories of ovarian morphology observed to coincide with the ovarian cycle. These categories were illustrated by representative

images randomly selected from sampling dates showing high occurrence of each category during the ovarian cycle (Figures 5.1 – 5.11). In all the illustrated images, the skin of the fish (located next to the position of the probe) was denoted by a solid white line; the periphery of the ovarian cross-section was denoted by a dashed white line; and ultrasound image artifacts caused by movement of the fish during image capture, by bone (vertebrae), or gas (airbladder) were identified with double headed arrows.

Echogenic Morphology of Ovaries Observed During the Recrudescence Phase. Three major types of ovarian echogenic morphology were observed with higher occurrences during the recrudescence period of the ovarian cycle and they were designated as Categories 1 (Undeveloped), 2 (Under-developed), and 3 (Developing) to denote sequential growth during the slow growth phase of the recrudescence period (Figures 5.1 – 5.4).

Category 1 was comprised of images which displayed ovarian development as a greyish, homogenous echogenic texture of a cross-sectional mass with visually discernible size variation. No oocytes were visible. Ovaries appeared small but were visually identifiable by the brighter echogenic structure in the periphery of the ovarian cross-section nearest the probe (Figure 5.1).

Category 2 (Under-developed) was defined by the first visible appearance of a heterogeneous echogenic pattern within the ovarian cross-section (Figure 5.2). This heterogeneity appeared as speckled texture against a darker background. This was the first detectable appearance of oocytes as grainy white speckles within the cross-section. Oocytes appeared with no

individually discernible form and shape, but collectively they contributed to the beginning of a more complex heterogeneous echogenic texture (Figure 5.2).

If the ovary was small, but no oocytes were visible in the image, the ovary was assessed as Category 1 (Undeveloped). If the ovary was small, but oocytes were visible as speckled structures within the ovary breaking the homogenous appearance and initiating a heterogeneous appearance, this ovary was assessed as Category 2 (Under-developed).

Category 3 (Developing) was developed based on an increased echogenic heterogeneity caused by the tissues and oocytes within a visibly enlarging ovary with a compacted appearance, and represented the period of recrudescence prior to fast growth of ovary and oocytes (Figure 5.3).

Echogenic Morphology of Ovaries Observed During the Spawning Phase. Four categories were developed to represent the biological transformations observed in the ovarian echogenic morphology during the spawning period.

Category 4 (Advanced) was created to represent ovaries and oocytes presumably in the fast growth period (late vitellogenesis) (Figure 5.5). Category 4 (Advanced) represented images of ovarian development in which a larger ovary appeared in close proximity to the skin relative to images in Category 3 (Developing), and the appearance of individually distinguishable oocytes (Figure 5.5).

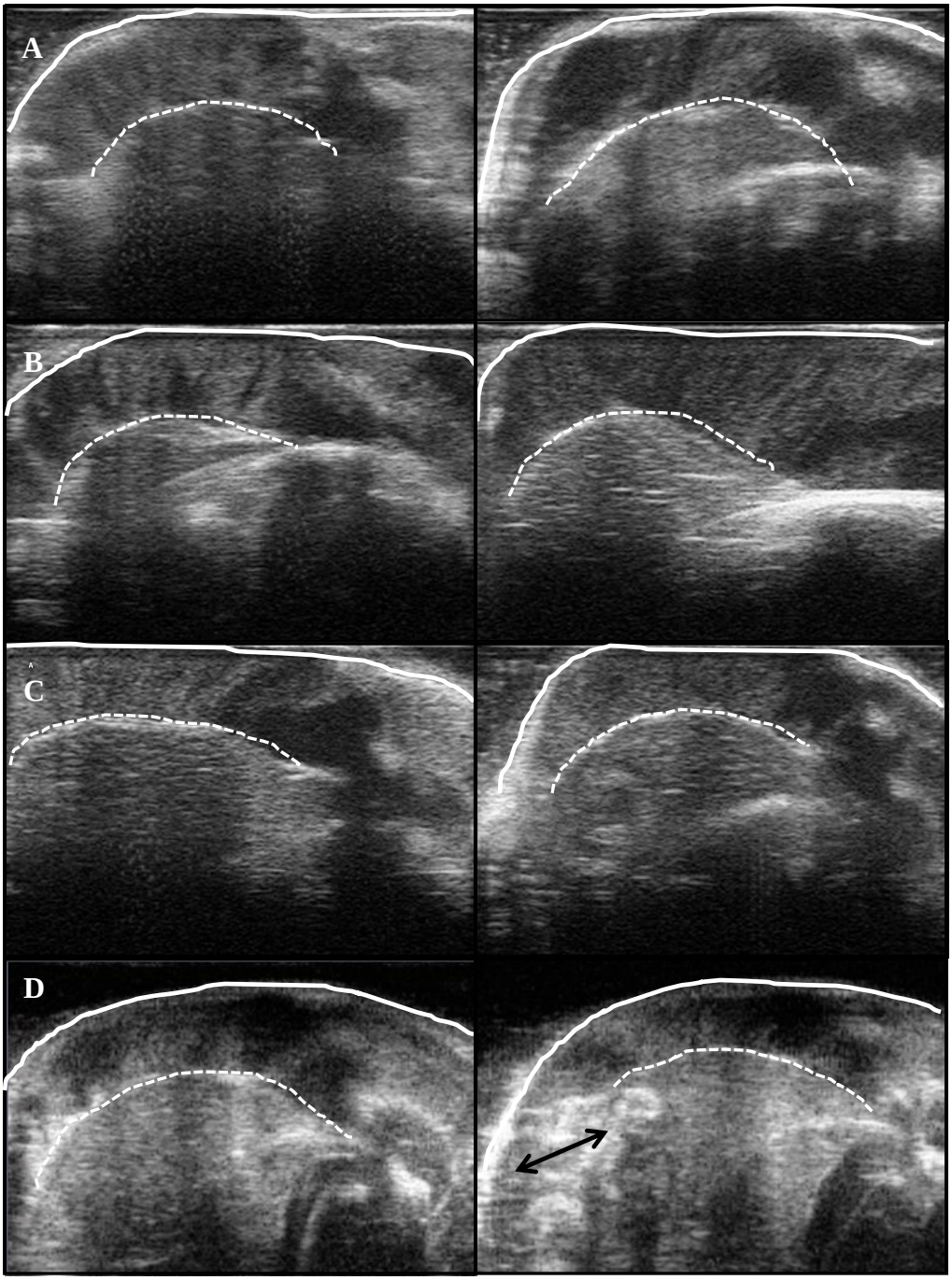


Figure 5.1 Category 1, Undeveloped. The paired horizontal images were randomly selected from ovarian assessments during recrudescence (October – March) on: (A) October 28, 2008 (n = 82 images); (B) November 5, 2008 (n = 89 images); (C) February 3, 2009 (n = 93 images); (D) March 25, 2010 (n = 30 images). The ovaries were visibly identifiable, and the ovarian cross-section appeared as a homogenous echogenic mass with a visible cross-sectional periphery in the near-field view and no visible oocytes. This morphology was best illustrated by ultrasound images in panel A.

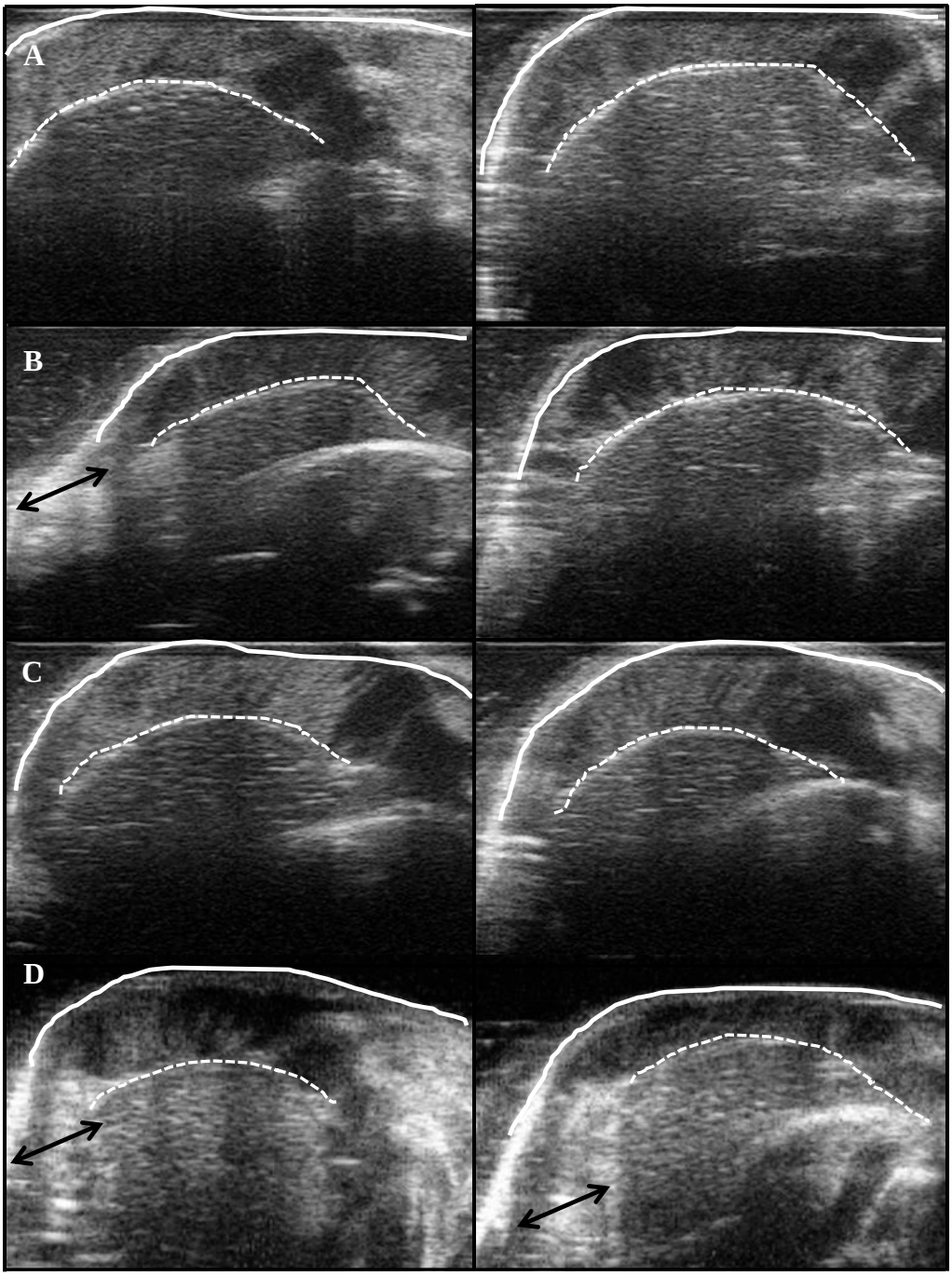


Figure 5.2 Category 2, Under-developed. The paired horizontal images were randomly selected from ovarian assessments during recrudescence (October – March) on: (A) October 28, 2008 (n = 10 images); (B) February 3, 2009 (n = 79 images); (C) March 13, 2009 (n = 56 images); (D) March 25, 2010 (n = 25 images). The ovaries were distinctly visible, encasing a heterogeneous echogenic texture within the cross-section which signaled the initiation of oocyte visibility within the image. Although the size of the ovaries varied, the most important echogenic feature was the initial appearance of oocytes, which appeared as specked bright echogenic granules with no shape or form embedded across a darker background, within the ovarian cross-section.

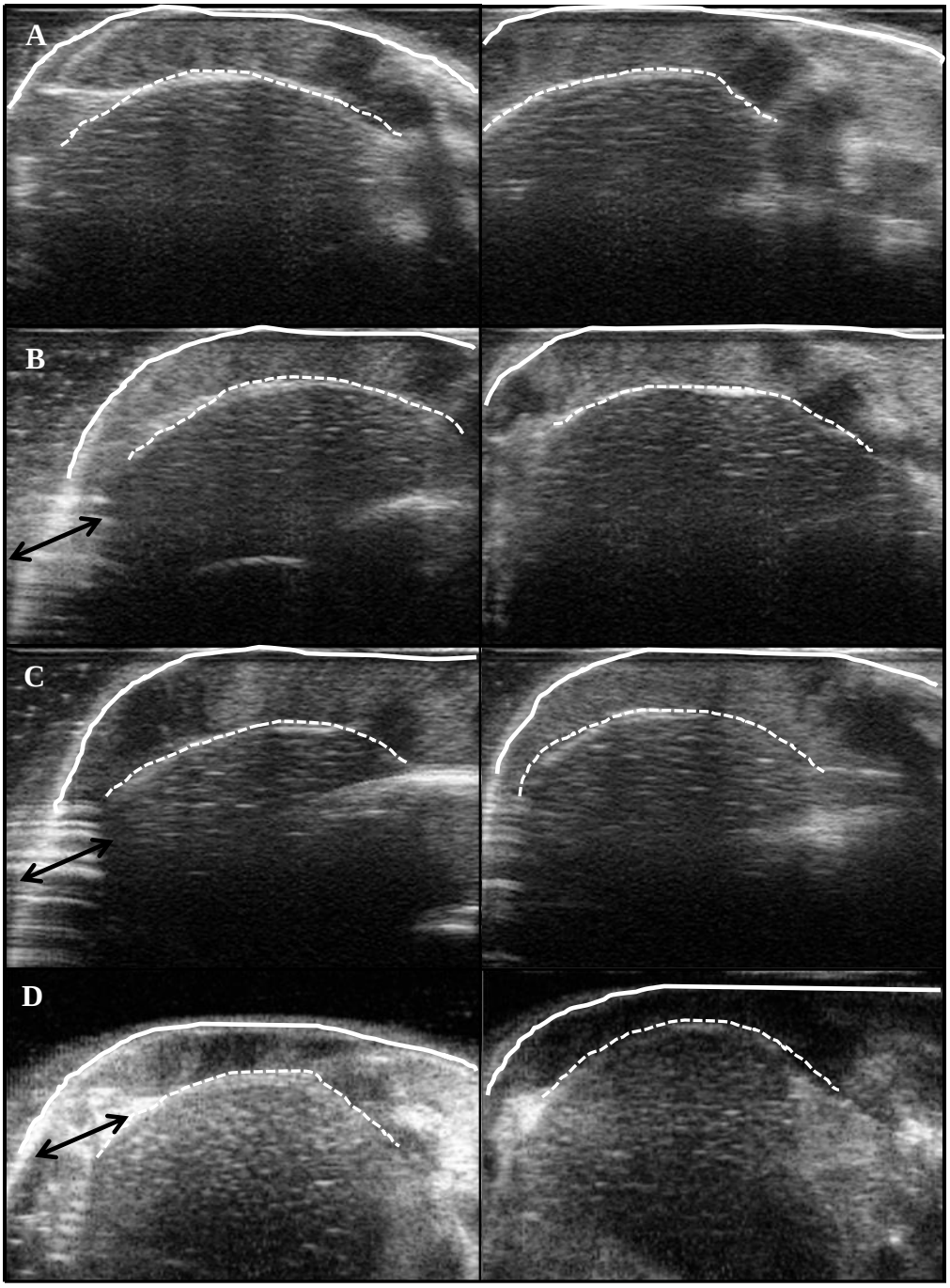


Figure 5.3 Category 3, Developing. The paired horizontal images were randomly selected from ovarian assessments during recrudescence (October – March) on: (A) October 28, 2008 (n = 5 images); (B) February 3, 2009 (n = 81 images); (C) March 13, 2009 (n = 55 images); (D) March 25, 2010 (n = 10 images). The ovary was highly discernible, visibly enlarging and appearing closer to skin; oocytes had a higher visibility as denoted by the increased echogenic heterogeneity within the ovarian cross-section; however, oocytes appeared small and compacted within the ovary.

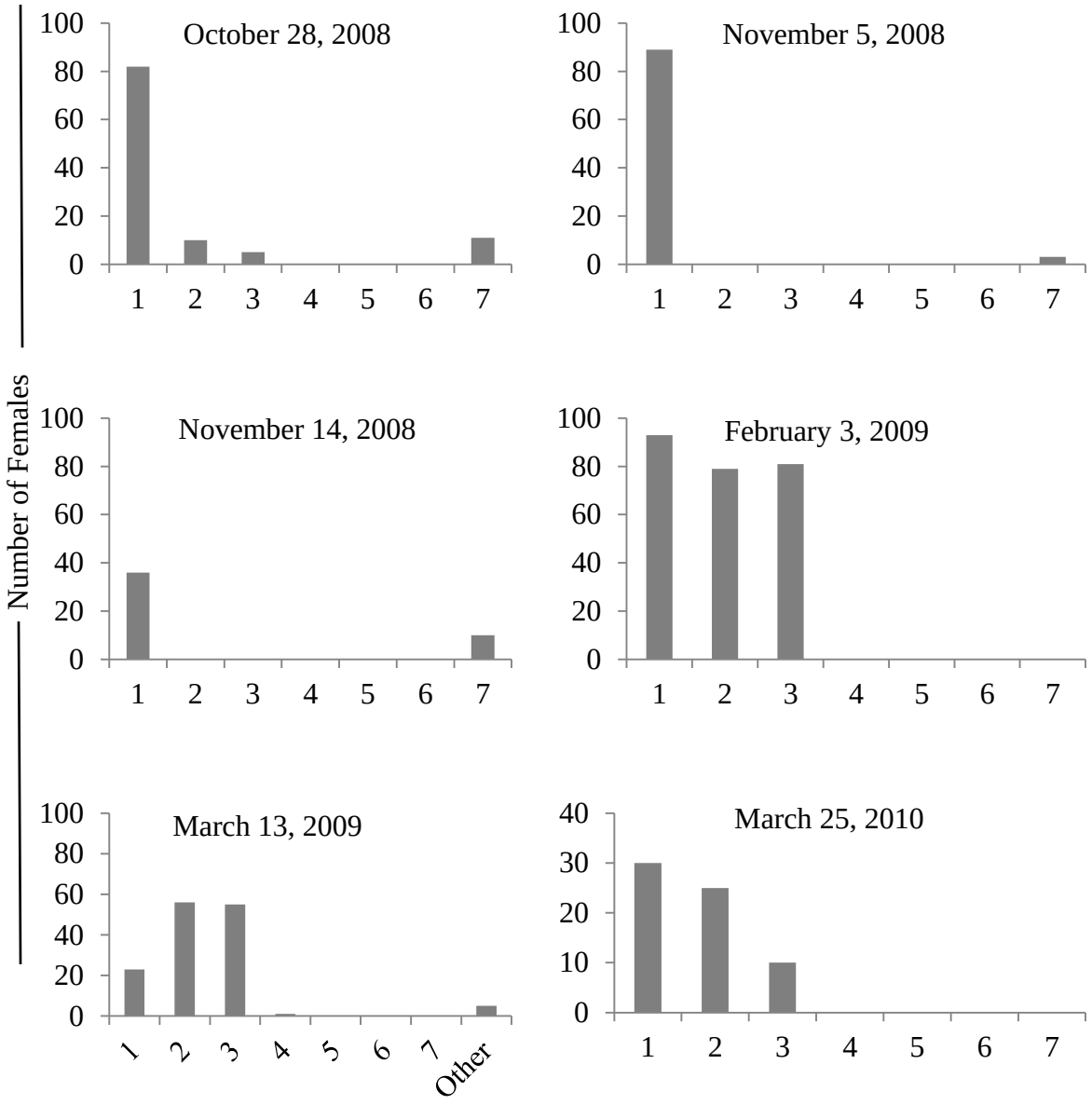


Figure 5.4 Ultrasound imaging ovarian development of channel catfish collected by seine during the recrudescence period of the reproductive cycle at the Aquaculture Research Station, Baton Rouge, LA. Category 1 (Undeveloped) occurred most frequently in October and November. This ovarian morphology designated as 'Undeveloped' was associated with early vitellogenesis and slow growth period of the ovarian cycle. Category 2 (Under-developed) and 3 (Developing) appeared most frequently in February and March prior to the initiation of the fast growth period of the ovarian cycle. The number of fish assessed = y-axis; the ultrasound imaging categorical assessments = x-axis. 'Other' represented a tentative intermediate categorical assessment of 3.5 during the development of the reproductive index. This attempt at defining a finer spectrum of categorical assessment was abandoned to create a classification assessment index based on easily recognizable biological stages of the ovarian cycle.

Category 5 (Mature) was created to represent ovaries and oocytes presumably ready for induced oocyte maturation (Figure 5.6). Category 5 represented images of ovaries which appeared with a highly organized and complex echogenic structure, with individual and enlarged oocytes discernible as a bright centripetal structure (cause by the yolk content of the oocytes) surrounded by a darker peripheral structure (caused by the cytoplasm and ovarian fluids at the periphery of the oocytes) (Figure 5.6).

Category 6 (Spawned) was created for ovaries presumably in a 'spent' state, i.e. ovaries that had undergone ovulation (Figure 5.7). Category 6 represented images of ovarian development in which ovaries appeared small and were difficult to distinguish from surrounding tissues with usually no oocytes visible (Figure 5.7).

Category 7 (Atretic) was created to represent ovaries presumably in an atretic state (Figure 5.8). Category 7 ovarian images appeared with an irregular internal echogenic texture which displayed ovaries and oocytes with an amorphic shape and gave the visual appearance of degeneration (Figure 5.8).

Hormone-Induced Spawning and Fertilization. Fish Injected for Artificial Spawning. Channel catfish ovarian development was assessed using ultrasound imaging prior to hormone injection, and fish in each category were selected for spawning trials during 2008 to 2010. During 2008 during preliminary development of the assessment index, of the 43 fish selected for injection, the majority (56%) of the ovarian images (i.e. 24 fish) were assessed as one of seven categories (Table 5.5). The remaining 44% of the 2008 ovarian image assessments (i.e. 19 of 43

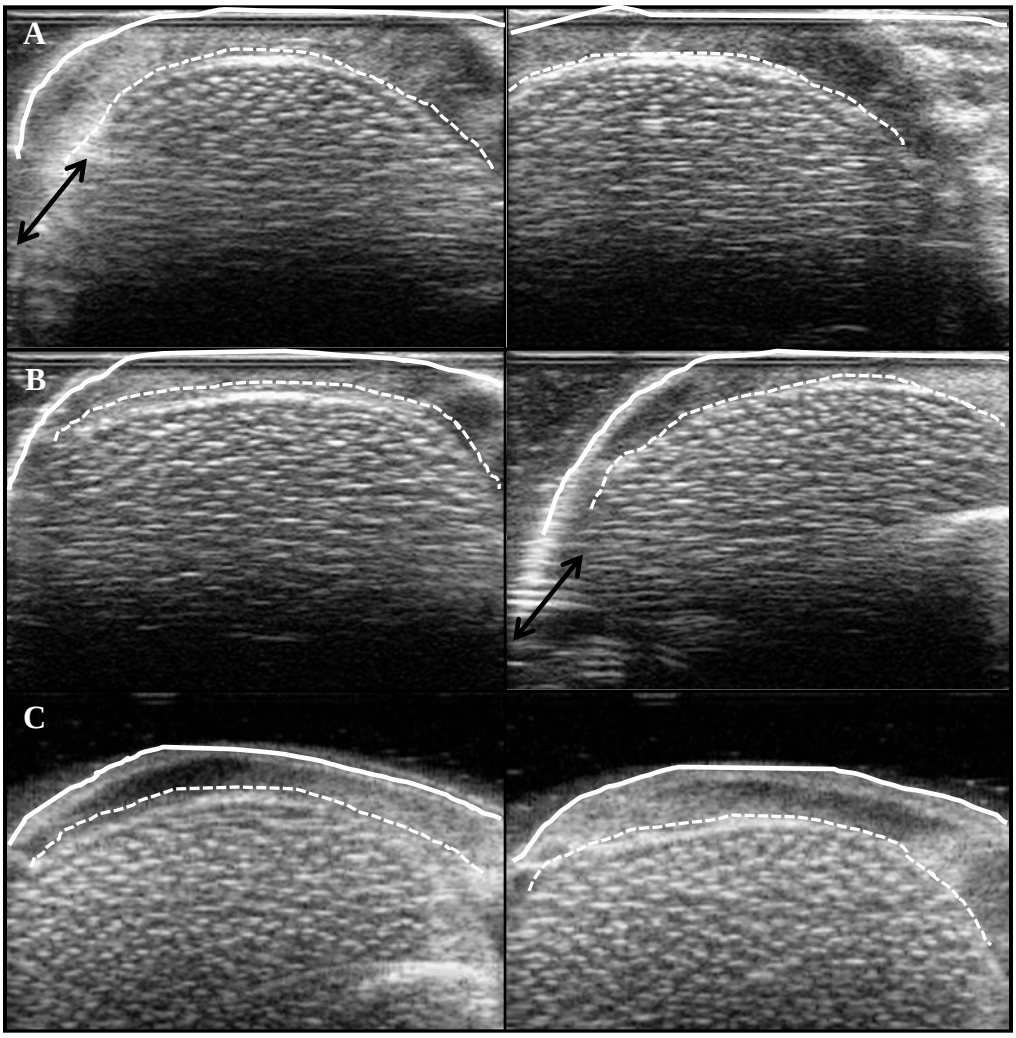


Figure 5.5 Category 4, Advanced. The paired horizontal images were randomly selected from ovarian assessments during spawning (April – May) on (A) April 25, 2009 (n = 4 images); (B) May 26, 2009 (n = 8 images); (C) May 5, 2010 (n = 26 images). The ovary appeared prominently within the displayed image, with the ovarian cross-sectional periphery closer to the skin surface and filled with individually distinguishable oocytes, which appeared in an enlarged and organized form throughout the ovary.

fish) were intermediate categories labeled as 'other' (Table 5.6). During 2009, of the 88 fish selected for injection, the majority (82%) of the ovarian images (i.e. 72 fish) were assessed as one of seven categories, while 16% (i.e. 13 of 84 fish) were intermediate tentative categories labeled as 'other' (Table 5.5 – 5.7). During 2010, 79 fish were selected for injection. The index was by then well characterized and all the fish were assessed using one of seven categories; no intermediate categories were used (Table 5.5, and 5.8).

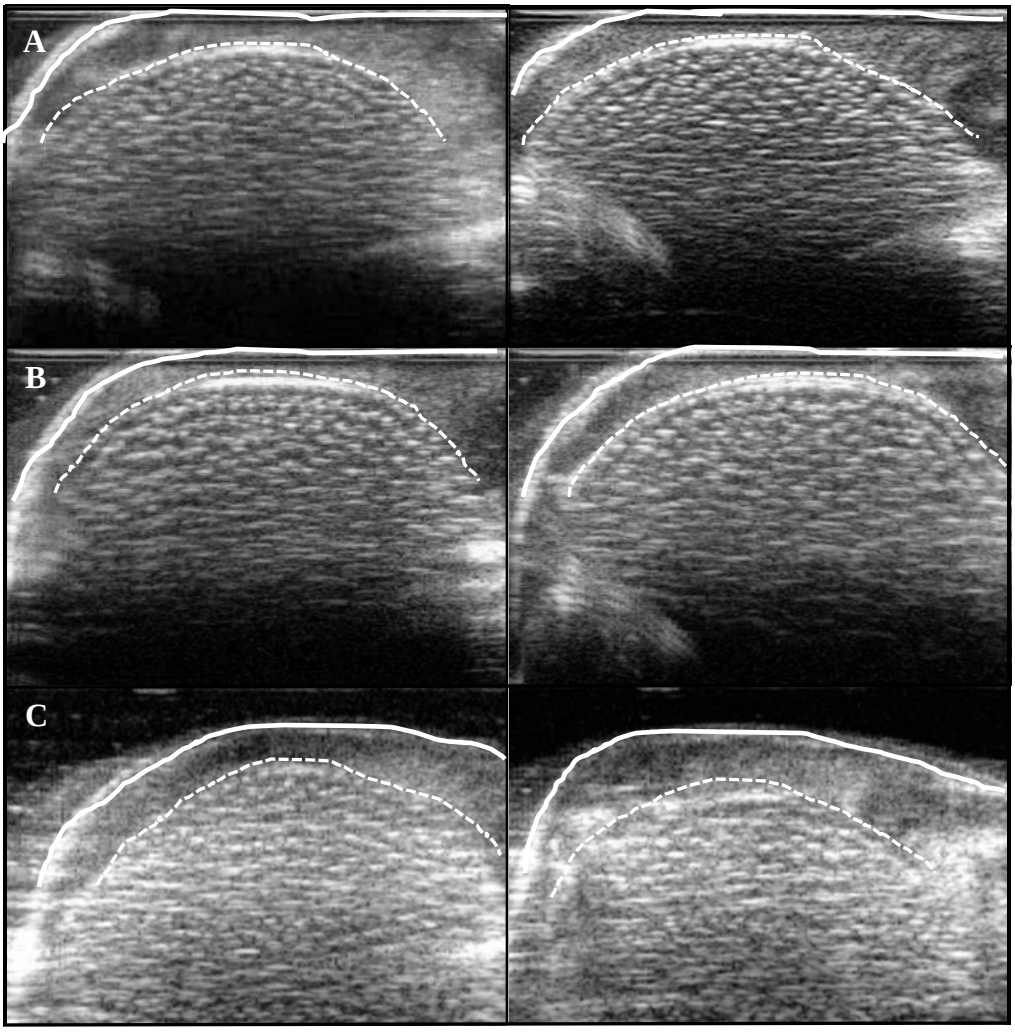


Figure 5.6 Category 5, Mature. The paired horizontal images were randomly selected from images recorded during spawning (April – May) on: (A) April 25, 2009 (n = 8 images); (B) May 26, 2009 (n = 25 images); (C) May 17, 2010 (n = 15 images). The ovary appeared extended with the closest proximity to the skin observed, and both ovary and oocytes dominated the displayed image. Oocytes were prominent, large, highly organized, with a discernible boundary.

The initial attempt to explore intermediate categories was discontinued during the development of the classification index because the categorical assessment was developed to identify readily discernible morphological changes that occurred during the annual ovarian cycle.

Fertilization. The ratio (fertilization estimate) of the average count of developing embryos to the average egg count in one monolayer for each female ranged from 0 to 0.2 during spawning

trials in 2008, from 0 to 0.6 during spawning trials in 2009, and 0 to 0.85 during spawning trials in 2010. There was no significant effect on the mean differences in fertilization caused by the spawning trials ($P = 0.9349$).

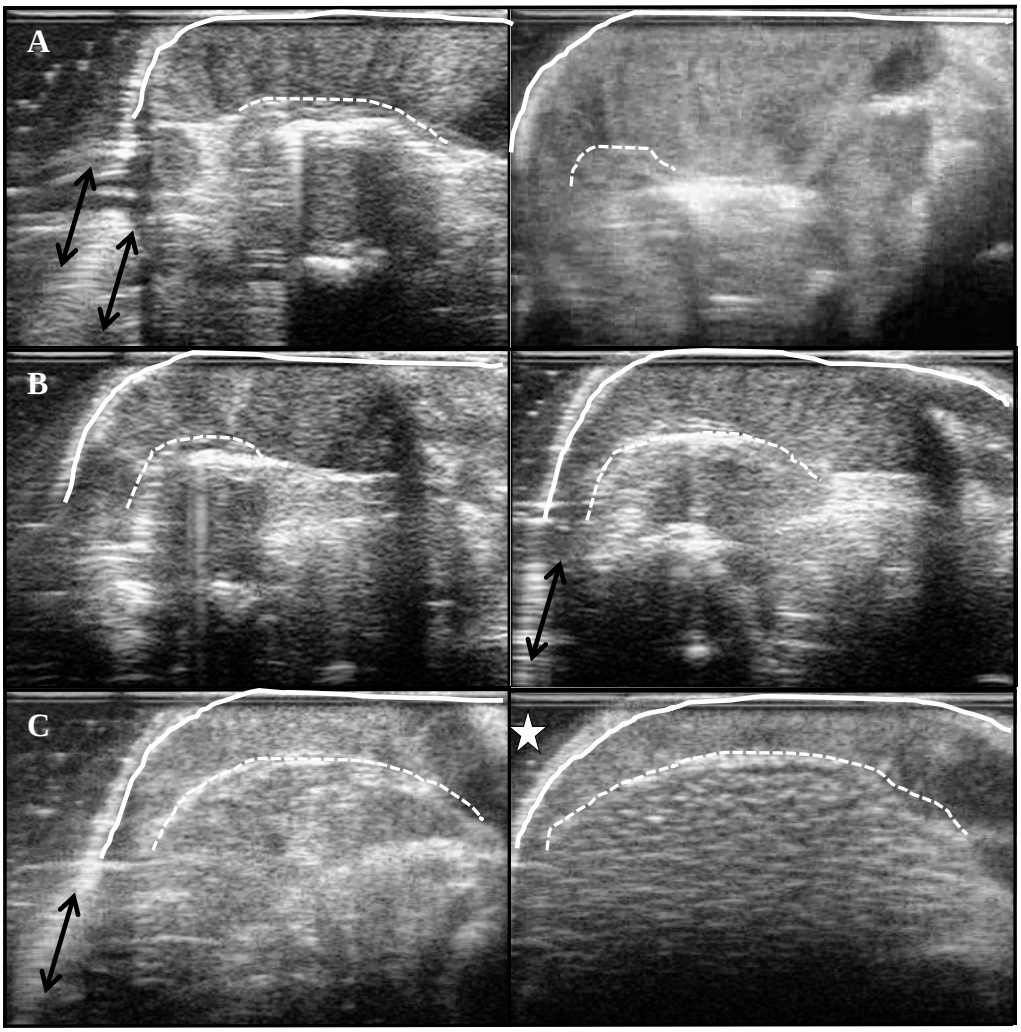


Figure 5.7 Category 6, Spawned (Spent). The paired horizontal images were randomly selected from images recorded during spawning on: (A) April 25, 2009 (n = 15 images); (B) May 9, 2009 (n = 14 images); (C) June 1, 2009 (n = 11 images). Ovaries appeared small and were barely distinguished against surrounding tissues with few or no oocytes visible. The image denoted by the star shape does not fit this definition and is presented to represent an assessment or data recording error.

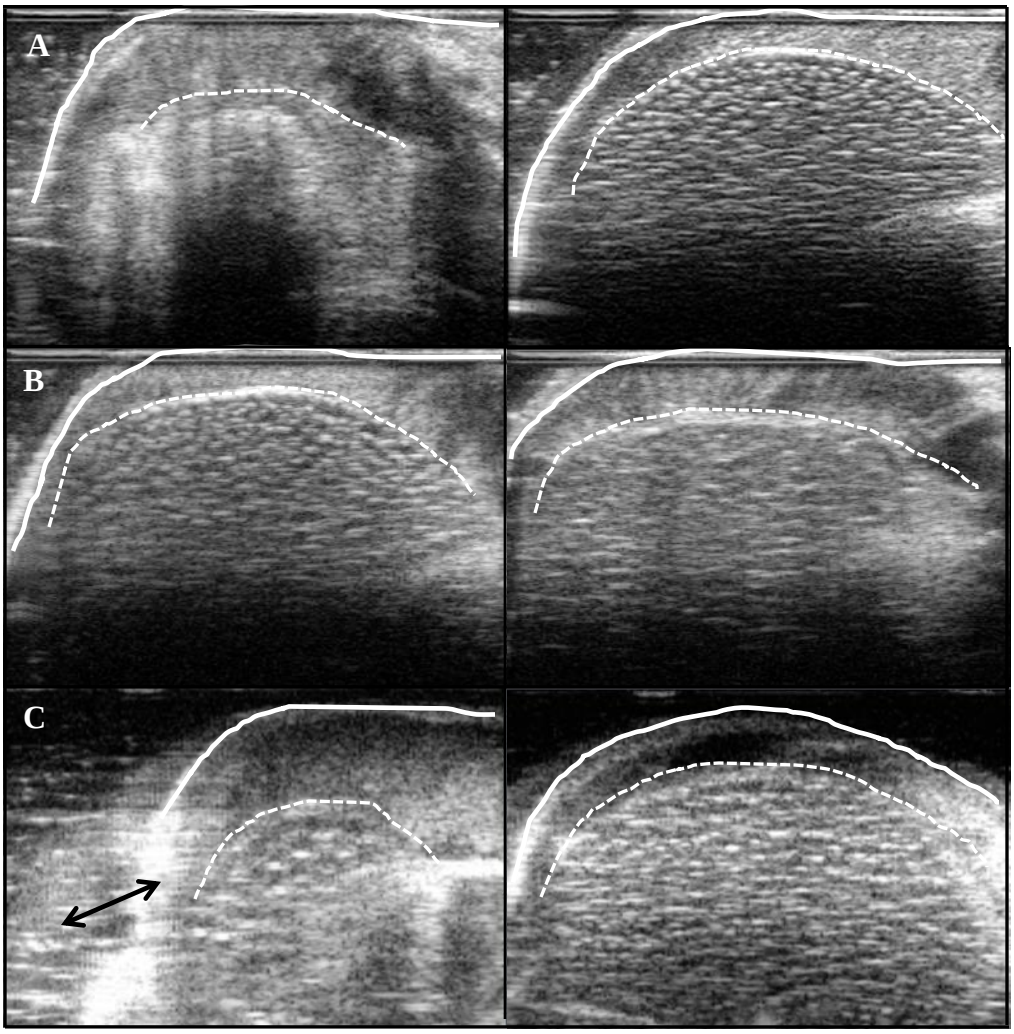


Figure 5.8 Category 7, Atretic. The paired horizontal images were randomly selected from images recorded late in the spawning season (May – June) on: (A) May 26, 2009 (n = 81 images); (B) June 1, 2009 (n = 54 images); (C) May 17, 2010 (n = 14 images). These images were of ovaries and oocytes that appeared irregular in echogenic appearance, and irregular and highly variable in shape and size. The double-headed arrow indicates a representative ultrasound image artifact generated by movement of the fish.

Accuracy of the Ultrasound Imaging Classification Assessments. The expected outcome for Categories 1, 2, 3, 6, and 7 was that no viable eggs would be collected during artificial spawning. The expected outcome for Categories 4 and 5 was that viable eggs would be collected during artificial spawning.

The percentage of the number of fish for which the predicted (expected) and actual (observed) biological outcomes were the same was calculated from the tabulated raw data on each of seven ultrasound imaging classification assessments during ten spawning trials (Table 5.6 – 5.8). Categories 1 (Undeveloped), 2 (Under-developed), and 6 (Spawned) had 100% agreement of the expected and observed biological outcome. Category 3 (Developing) had 78 to 100% agreement of the expected and observed biological outcome.

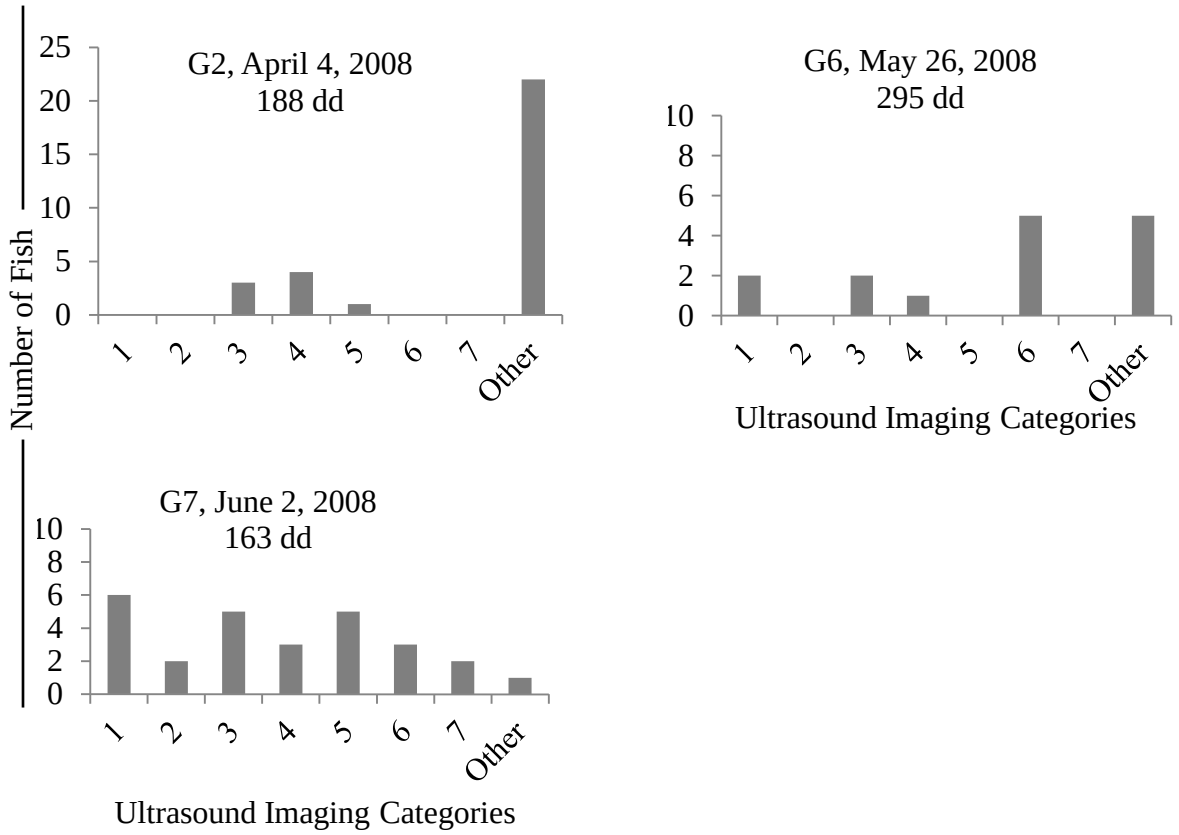


Figure 5.9 Ultrasound imaging ovarian development assessments of channel catfish collected by seine from three ponds during 2008 spawning trials. The degree-day (dd) measurements for thermal reproductive conditioning were within (G2, G7 ponds) or greatly exceeded (G6 pond) the window (150 – 172 degree-days) for the conclusion of spawning based on guidelines by Pawiroredjo et al. 2008. Data reported as 'Other' represented tentative intermediate categorical assessments during the earliest part of the development of the reproductive index. "Other' included fish that were assigned intermediate assessments of 3.5 to 4.5 and images in which the ovary was not visible due to ultrasound artifacts caused by visceral fat. This attempt at defining a finer spectrum of categorical assessment was coalesced to create a classification assessment index based on easily recognizable biological stages of the ovarian cycle.

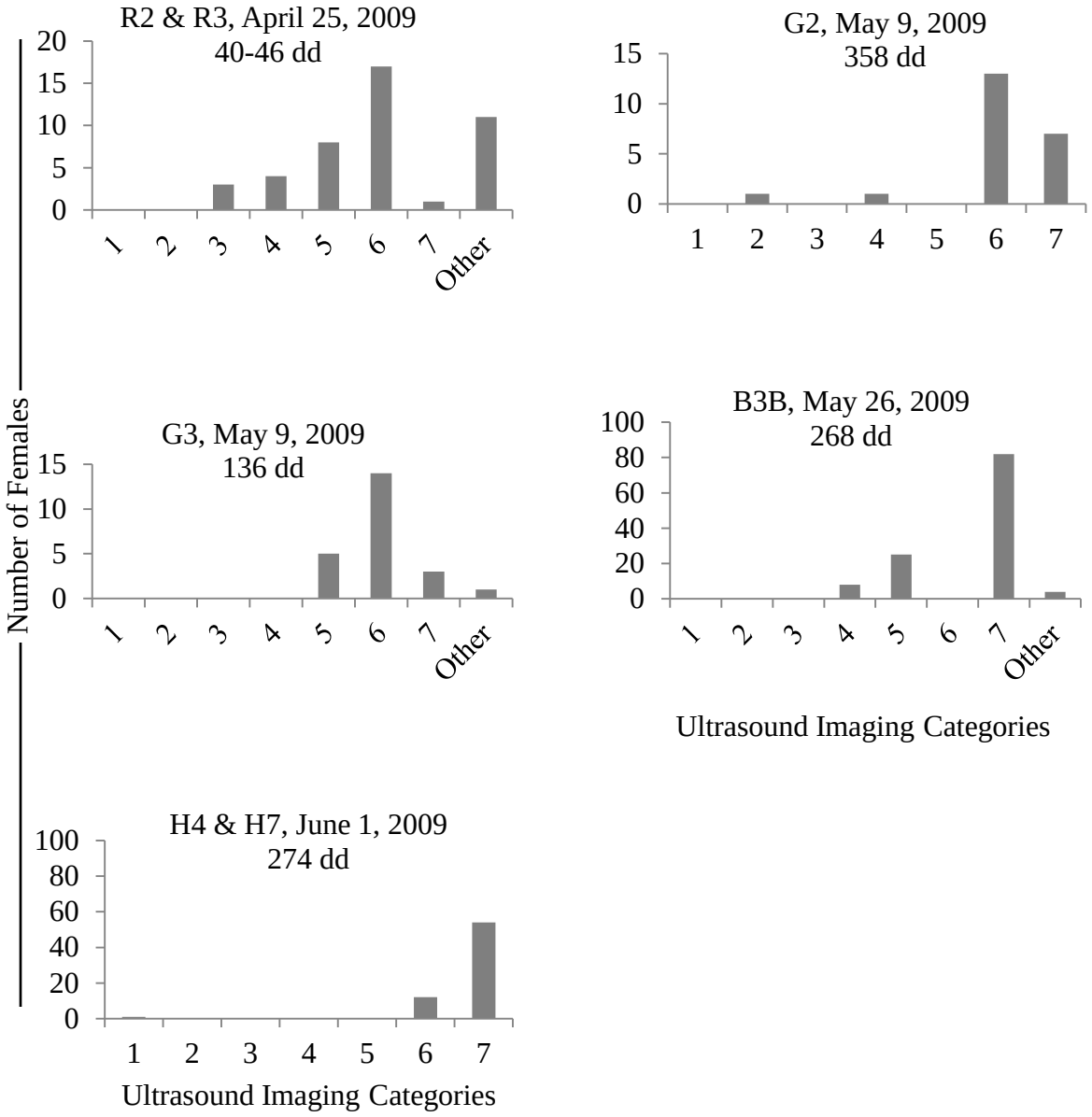


Figure 5.10 Ultrasound imaging ovarian development assessments of channel catfish collected by seine from ponds used in 2009 spawning trials. Ponds R2 and R3 had a reproductive thermal conditioning profile considered before the onset of spawning (57 – 81 degree-days). Pond G3 was above the median of the spawning period (99 – 129 degree-days). Ponds G2, B3B, H4 and H7 greatly exceeded the degree-day guidelines defined for the conclusion of spawning (150 – 172 degree-days) and ovarian development assessed as spawned (Category 6) and atretic (Category 7) were at their highest incidence of occurrence in these ponds. Data reported as 'Other' represented tentative intermediate categorical assessments during the development of the reproductive index. "Other' included fish which were assigned intermediate assessments of 3.5 and 4.5.

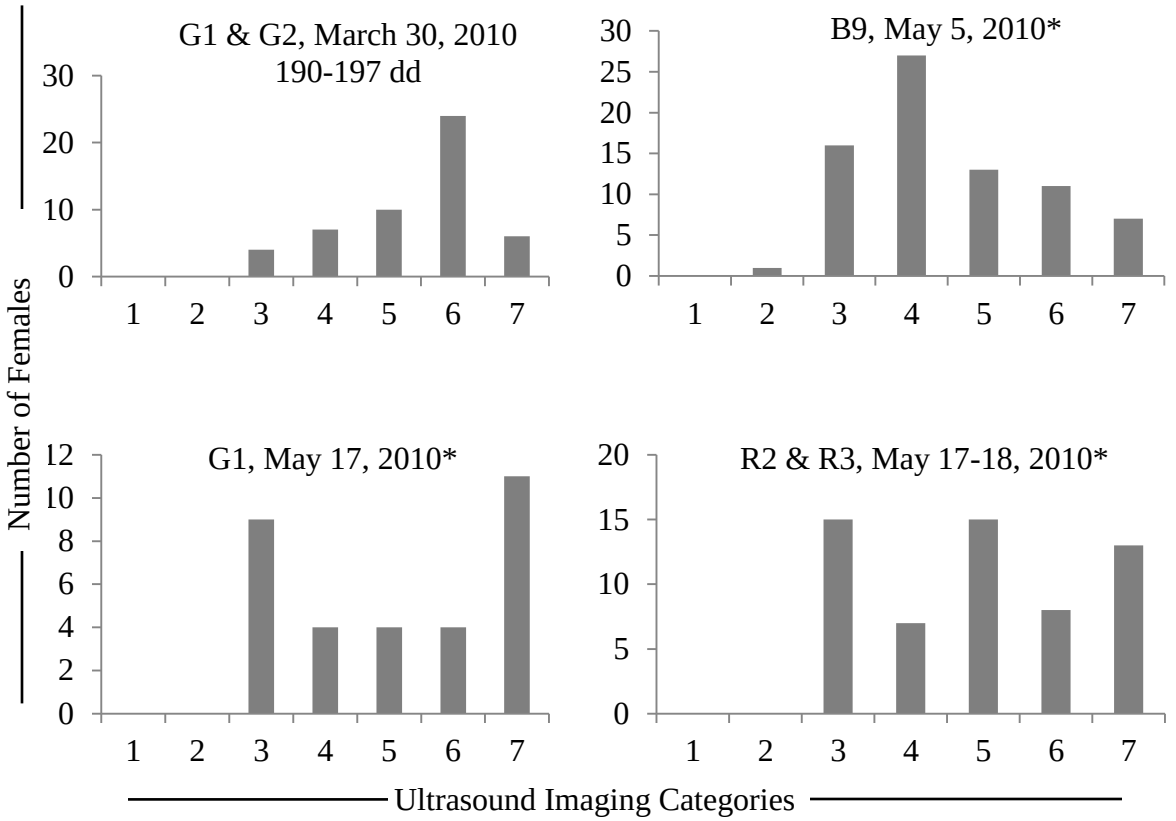


Figure 5.11 Ultrasound imaging ovarian development assessments of channel catfish collected by seine from ponds used during 2010 spawning trials. The degree-days calculated for ponds G1 and G2 exceeded the window of the conclusion of spawning (150 – 172 degree-days). *Temperature data were not available for two spawning trials.

Table 5.5 During the development of the ultrasound imaging classification system preliminary attempts at defining intermediate categories (Other) to microanalyze morphological features between images were discontinued to develop a system in which ovarian development would be easily interpreted and assessed based on the ovarian cycle morphological transformations.

Ultrasound Imaging	Number of Fish Injected			
Categories	2008	2009	2010	Total
1. Undeveloped	1	0	0	1
2. Under-developed	1	1	0	2
3. Developing	9	3	9	21
4. Advanced	7	11	24	42
5. Mature	5	34	32	71
6. Spawned	1	4	3	8
7. Atretic	0	19	11	30
Other (See Table 5.6, 5.7)	19	16	0	35
Total	43	88	79	210

Category 4 (Advanced) had 25 to 43% agreement of the expected and observed biological outcome. Category 5 (Mature) had 38 to 60% agreement of the expected and observed biological outcome. Category 7 (Atretic) had 42% (2009) and 82% (2010) agreement of the expected and observed biological outcomes.

Statistical analysis of the tabulated data for each category in each spawning trial (Table 5.6 – 5.8) showed a significant effect ($P < 0.002$) of the expected outcome on the observed outcome. That is, changes in the expected value changed the observed value. The year and the interaction between year and expected outcome had no significant effect ($P > 0.05$) on the observed outcome.

Accuracy of Classifications Based on the Error Matrix. An error matrix was generated from the predicted values in the output of the previous statistical analysis to test the individual relationships of the expected and observed outcome for each ultrasound imaging assessment category (Table 5.9). The probability of correct assessments in the error matrix was based on the agreement of the expected and observed outcomes compared for each category and to each other.

The probability of a correct assessment (i.e. the expected and observed outcome was the same) for Category 1 (Undeveloped) was very likely (0.99 probability). That is, fish with an ovarian development assessment of Category 1 would very likely not produce viable eggs. The expected outcome of Category 1 could be confused (0.99 probability) with the observed outcome of Category 2, but not (0 probability) with the observed outcomes for Categories 3, 4, 5, 6 and 7.

Table 5.6 The number of fish injected (Inj.) and the number of fish for which the expected and observed outcomes were the same (Obs.) were listed for three artificial spawning trials in 2008. The percent (%) agreement of the observed and expected outcome (Total Observed/Total Injected x 100) was calculated for seven ultrasound imaging categorical assessments of channel catfish ovarian development. The expected outcome for Categories 1, 2, 3, 6, and 7 was that no viable eggs would be collected, and for Categories 4 and 5 that viable eggs would be collected. The data for categorical assessments 1, 2, 3, 4, 5, 6, and 7 for each trial were used in the statistical analysis comparing the expected to observed outcome.

Ultrasound imaging categorical assessments of ovarian development	Trial I Number of fish		Trial II Number of fish		Trial III Number of fish		Total	Total	% agreement of expected and observed
	Inj.	Obs.	Inj.	Obs.	Inj.	Obs.	Inj.	Obs.	
1. Undeveloped	-	-	1	1	-	-	1	1	100
2. Under-developed	-	-	-	-	1	1	1	1	100
3. Developing	3	3	1	1	5	5	9	9	100
4. Advanced	4	3	-	-	3	0	7	3	43
5. Mature	1	0	-	-	4	3	5	3	60
6. Spawned	-	-	1	1	-	-	1	1	100
7. Atretic	-	-	-	-	-	-	-	-	-
*'Other'									
3.5	1	0	4	2	1	1	6	3	
3.75	2	0	-	-	-	-	2	0	
3.75-4	4	2	-	-	-	-	4	2	
4.5	3	**Note	-	-	-	-	3	-	
not visible (e.g. visceral fat)	4	0	-	-	-	-	4	0	

*'Other' = exploratory intermediate categorical assessments were discontinued but reported as an artifact of the developmental process of the classification system; 'Observed' for 'Other' = number of fish from which viable (fertilizable) eggs were collected.

**Note: These three fish were selected to undergo atresia; no egg collection and fertilization data were recorded.

Table 5.7 The number of fish injected (Inj.) and the number of fish for which the expected and observed outcomes were the same (Obs.) were listed for four spawning trials in 2009. The percent (%) agreement of the observed and expected outcome (Total Observed/Total Injected x 100) was calculated for seven ultrasound imaging categorical assessments of channel catfish ovarian development. The expected outcome for Categories 1, 2, 3, 6, and 7 was that no viable eggs would be collected, and for Categories 4 and 5 that viable eggs would be collected. The data for categorical assessments 1, 2, 3, 4, 5, 6, and 7 for each trial were used in the statistical analysis comparing the expected to observed outcome.

Ultrasound imaging categorical assessments of ovarian development	Trial IV Number of fish		Trial V Number of fish		Trial VI Number of fish		Trial VII Number of fish		Total	Total	% agreement of expected and observed
	Inj.	Obs.	Inj.	Obs.	Inj.	Obs.	Inj.	Obs.	Inj.	Obs.	
1. Undeveloped	-	-	-	-	-	-	-	-	-	-	-
2. Under-developed	-	-	1	1	-	-	-	-	1	1	100
3. Developing	3	3	-	-	-	-	-	-	3	3	100
4. Advanced	4	1	1	1	6	1	-	-	11	3	27
5. Mature	8	3	5	1	21	9	-	-	34	13	38
6. Spawned	1	1	-	-	-	-	3	3	4	4	100
7. Atretic	-	-	2	1	2	1	15	6	19	8	42
*'Other'											
3.5	11	4	1	1	3	1	-	-	15	6	
4.5	-	-	-	-	1	0	-	-	1	0	

*'Other' = exploratory intermediate categorical assessments were discontinued but reported as an artifact of the developmental process of the classification system; 'Observed' for 'Other' = number of fish from which viable (fertilizable) eggs were collected.

The probability of correct assessment of Category 2 (Under-developed) was very likely (0.93 probability). That is, fish with an ovarian development assessment of Category 2 would very likely not produce viable eggs. The expected outcome of Category 2 could be confused (0.99 probability) with the observed outcome of Categories 3 and 4, but not (0 probability) with the observed outcome of Categories 5, 6, and 7.

Table 5.8 The number of fish injected (Inj.) and the number of fish for which the expected and observed outcomes were the same (Obs.) were listed for four spawning trials in 2010. The percent agreement of the observed and expected outcome (Total Observed/Total Injected x 100) was calculated for seven ultrasound imaging categorical assessments of channel catfish ovarian development. The expected outcome for Categories 1, 2, 3, 6, and 7 was that no viable eggs would be collected, and for Categories 4 and 5 that viable eggs would be collected. All the data for categorical assessments for each trial were used in the statistical analysis comparing the expected to observed outcome.

Ultrasound imaging categorical assessments of ovarian development	Trial VIII Number of fish		Trial IX Number of fish		Trial X Number of fish		Total	Total	% agreement of expected and observed
	Inj.	Obs.	Inj.	Obs.	Inj.	Obs.	Inj.	Obs.	
1. Undeveloped	-	-	-	-	-	-	-	-	-
2. Under-developed	-	-	-	-	-	-	-	-	-
3. Developing	3	3	3	2	3	2	9	7	78
4. Advanced	6	2	7	1	11	3	24	6	25
5. Mature	7	4	7	3	18	9	32	16	50
6. Spawned	-	-	3	3	-	-	3	3	100
7. Atretic	8	7	1	1	2	1	11	9	82

The probability of correct assessment of Category 3 (Developing) was very likely (0.94 probability). That is, fish with an ovarian development assessment of Category 3 would very likely not produce viable eggs. The expected outcome of Category 3 could be confused (0.99 probability) with the observed outcome of Categories 4 and 5, but not (0 probability) with the observed outcome of Categories 6, and 7. The probability of correct assessment of Category 4 (Advanced) was likely (0.86 probability). That is, fish with an ovarian development assessment of Category 4 would likely produce viable eggs. The expected outcome of Category 4 could be confused (0.99 probability) with the observed outcome of Categories 5 and 6, but not (0 probability) with the observed outcome of Categories 1, 3, and 7.

Table 5.9 Error matrix for ultrasound imaging categories comprising the reproductive development index during 2008-2010. The probability of correct assessment (Mu) was calculated for each expected outcome based on observed outcomes of each ultrasound imaging categorical assessment during the spawning trials. The expected outcome of Categories 1, 2, 3, 6 and 7 was that no viable eggs would be collected. The expected outcome of Categories 4 and 5 was that viable eggs would be collected.

Expected	Observed	Mu	Summary of Interpretation
1	1	0.99389	The expected and observed outcome for Category 1 was very likely the same.
1	2	0.99996	The expected outcome for Category 1 could be confused with Category 2 observed outcome.
1	3	0	
1	4	0	The expected outcome for Category 1 would in all likeliness not be confused with
1	5	0	the observed outcomes of Categories 3, 4, 5, 6 and 7.
1	6	0	
1	7	0	
2	1	0.07837	
2	2	0.92554	The expected and observed outcome for Category 2 was likely the same.
2	3	0.99975	The expected outcome for Category 2 could be confused with
2	4	0.99999	the observed outcome for Category 3 and 4.
2	5	0	The expected outcome for Category 2 would in all likeliness
2	6	0	not be confused with Categories 5, 6 or 7.
2	7	0	
3	1	0.00035	
3	2	0.04865	
3	3	0.94235	The expected and observed outcome for Category 3 was likely the same.
3	4	0.99841	The expected outcome for Category 3 could be confused with the
3	5	0.99998	observed outcome of Categories 4 or 5.
3	6	0	The expected outcome for Category 3 would in all likeliness not be confused with the
3	7	0	observed outcome of Categories 6 or 7.
4	1	0	
4	2	0.0005	
4	3	0.13835	

Table 5.9 Continued.

Expected	Observed	Mu	Summary of Interpretation
4	4	0.86047	The expected and observed outcome for Category 4 was likely the same.
4	5	0.9975	The expected outcome for Category 4 could be confused
4	6	0.99995	with the observed outcome of Categories 5 or 6.
4	7	0	The expected outcome for Category 4 would very likely not be confused
5	1	0	with observed outcome of Categories 1, 2, 3 or 7.
5	2	0.00001	
5	3	0.00326	
5	4	0.11153	
5	5	0.89043	The expected and observed outcome for Category 5 was likely the same.
5	6	0.99747	The expected outcome for Category 5 could be confused with observed outcome of Category 6.
5	7	0	The expected outcome for Category 5 would very likely not be confused with the observed outcome of Categories 1, 2, 3, 4 and 7.
6	1	0	
6	2	0	
6	3	0.00006	The expected outcome for Category 6 would very likely not be confused with the observed
6	4	0.0022	outcome of Categories 1, 2, 3, 4, 5 and 7.
6	5	0.1248	
6	6	0.87351	The expected and observed outcome for Category 6 was likely the same.
6	7	0	
7	1	0	The expected outcome for Category 7 would in all likeliness not be confused with the
7	2	0	observed outcome of Categories 1, 2, and 3.
7	3	0	
7	4	0.00001	The expected outcome for Category 7 would not likely be confused with the
7	5	0.00058	observed outcome of Categories 4, 5 and 6
7	6	0.02753	
7	7	1	The expected and observed outcome for Category 7 was the same.

The probability of correct assessment of Category 5 (Mature) was likely (0.89 probability). That is, fish with an ovarian development assessment of Category 5 would likely produce viable eggs. The expected outcome of Category 5 could be confused with the observed outcome of Category 6 (0.99 probability) and Category 4 (0.11 probability), but not (0 probability) with the observed outcome of Categories 1, 2, 3 and 7.

The probability of correct assessment of Category 6 (Spawned) was likely (0.87 probability). That is, fish with an ovarian development assessment of Category 6 would likely not produce viable eggs. The expected outcome of Category 6 could be confused (0.13 probability) with the observed outcome of Category 5, but not (0 probability) with the observed outcome of Categories 1, 2, 3, 4 and 7.

The probability of correct assessment of Category 7 (Atretic) was likely (1.0 probability). That is, fish with an ovarian development assessment of Category 7 would likely not produce viable eggs. The expected outcome of Category 7 could be confused (0.02 probability) with the observed outcome of Category 6, but not (0 probability) with the observed outcome of Categories 1, 2, 3, 4 and 5.

Discussion

Development of the Ultrasound Imaging Classification Index. The ultrasound images varied throughout the ovarian cycle and within specific periods of the recrudescence and spawning phases, representing the ongoing growth and continuous process of ovarian development from early to late vitellogenesis. During the early development of ultrasound imaging reproductive index, tentative intermediate categories were created (Table 5.6, 5.7). The first year was the

highest percentage of 'other' intermediate categories created in an attempt to classify the image morphology into a finer spectrum. However, the goal was to create a simplified, easy-to-use tool for assessment of ovarian morphology. No attempt was made to re-assess images of intermediate categories because these were tentative assignments representing 17% of the total ultrasound imaging assessments and because real-time imaging ensures optimal condition for assessment. For example, artifacts that may be generated during image capture and ultrasonography (by recording of a still image from a video) or artifacts created by internal anatomy (e.g. airbladder) may be avoided and eliminated. As a second option, images of ovaries may be assessed in videos which include a complete scan of the ovary, and finally positioning the probe alongside the dorsal fin around the center of the longitudinal side of the ovary for 2 to 3 s to view and assess the echogenic morphology displayed. The disadvantage of using still images for assessment is that depending on the way the image was captured or recorded, ultrasound image artifacts that may disrupt assessment can be introduced.

The Conceptual Base for the Ultrasound Imaging Reproductive Index. The development of an ultrasound assessment index was based on the conceptual framework of ovarian development representative of the recrudescence period (ovarian and oocyte growth leading to maturation and ovulation) and the spawning period during the reproductive cycle of adult channel catfish so that each category showed corresponding echogenic morphology of visible sequential growth and changes occurring during the ovarian cycle. The goal was to identify visually observable ovarian echogenic morphology to develop discrete, easily recognizable categories where the numerical order and biological descriptor (name of the category) was representative of ovarian biological transformations during the reproductive cycle.

These categories needed to be relevant to the needs of commercial hatcheries to make decisions for hormone injections. Category 1 (Undeveloped) was used for ovarian images of which were presumed to be in the initial stages of oocyte development, and therefore the least developed reproductive state; Category 2 (Under-developed) was considered representative of more advanced development than Category 1 but less developed than Category 3 (Developing). Category 4 (Advanced) were considered to be at a more advanced reproductive state than Category 3 but less advanced than Category 5 (Mature), which was presumed to be the most advanced state of ovarian reproductive development leading to oocyte maturation and ovulation.

During the spawning period of channel catfish two markedly different morphologies have been reported to appear: large ovaries with peak gonadosomatic index (GSI) values of pre-spawn females (9 to 15%) and small ovaries with the smallest GSI values (0.05- 0.06%) of spawned females in April through July (MacKenzie et al. 1989; Banks et al. 1999). During the spawning sampling period in the present study, spawned ovaries of small shape and size were observed as well as large ovaries with oocytes losing their structural integrity and appearing as disintegrating. Although a spent ovary may be atretic especially if it was recently ovulated and has remnant follicles, based on the known ovarian morphological changes associated with spawning and ovulation, and on atretic processes involving resorption of all oocytes (no spawning occurred), two other major categories were created to systematically assess ovaries presumed to be spent or visibly atretic. Category 6 (Spawned) was created to represent a spent reproductive state appearing as a small (difficult to discern) ovary completely devoid of oocytes. Category 7 (Atretic) was created to represent regressing ovaries and oocytes that were easily visible, and highly irregular in shape, size and echogenic appearance. Category 7 was directly related to

decision-making in selecting which females to inject with hormones since this direct view of the ovary may include fish which may appear with the external morphological characteristics of a swollen abdomen that is soft and protruding. A direct view of internal morphology of this type of ovarian development would enable elimination of such fish from artificial spawning and thus improve efficiency of hatchery reproduction. Fish in either Category 6 or Category 7 should not be injected with hormones to collect viable eggs.

Categorical organizations that provide a general and useful overview of continuous and highly complex processes are used to understand biological systems, such as the conventional stages assigned for describing mitosis. The four main mitotic divisions are prophase, metaphase, anaphase and telophase. Because of the continuous and complex nature of mitotic division, there are processes and sequences of events that overlap during the transition between the main phases describing mitosis. For instance, chromosome condensation (coiling structures) begins during prophase and transitions (a period called 'prometaphase') into metaphase after condensation ends for chromosome alignment. Similarly, the process of ovarian development is a highly complex continuous process, and the ultrasound imaging categories describe major morphological processes observed during the ovarian cycle. The classification system, therefore, was intended to organize highly variable morphology caused by complex biological processes based on the ultrasound image morphology to provide a framework for interpretation of ovarian development.

Hormone-Induced Spawning and Fertilization. Few fish (n = 3 total) were injected to induce spawning in Categories 1 and 2 primarily because these fish had small ovaries, no visible oocytes, and any attempt to artificially spawn these fish would not produce viable eggs. No

attempt was made to artificially spawn any fish reproductively conditioned at ambient temperatures in these two categories in October through March when the frequency was highest (Figure 5.4), and the frequency of these two categories were less during the spawning periods (Figure 5.9 – 5.11). In general, the estimated fertilization rate of viable eggs was of low quality (i.e. defined as less than 50%) (Bates and Tiersch 1998). The thermal profile data of the ponds indicate that fish were held for a longer period of time in ambient and geothermally regulated ponds than was optimal for obtaining broodstock for viable egg production. The degree-day profiles of half (8 of the 16 ponds) of the ponds (Figure 5.9 – 5.11) used in reproductive conditioning were beyond the recommended degree-day guidelines recommended for the conclusion of spawning (150 – 172 dd) during which 90% of the broodstock were expected to have spawned (Pawiroredjo et al. 2008). For example, the degree-day profiles were 188 dd (pond G2), 295 dd (pond G6), 398 dd (pond G2), 296 dd (pond B3B), 274 dd (ponds H4 and H7), and 190 to 197 dd (ponds G1 and G2) for ponds used in Spawning Trials I, II, V, VI, VII, and XIII. This may have reduced overall egg quality, but did not interfere with ultrasound assessments.

Accuracy of the Ultrasound Imaging Classification Assessments. The accuracy of the ultrasound imaging assessments was assessed based on the comparison of the expected to the observed outcome of either viable or unviable egg production for each category during hormone-induced spawning. Categories 1 (Undeveloped), 2 (Under-developed) and 3 (Developing) characterized ovarian development that coincided with early vitellogenesis and slow growth during the early recrudescence phase. The expected outcome was that fish assessed as Categories 1, 2 and 3 would produce no eggs, and that if eggs were collected, they would be

unviable. Categories 4 (Advanced) and 5 (Mature) characterized ovarian development observed during the spawning phase and were associated with late vitellogenesis and fast growth prior to ovulation. The expected outcome was that fish assessed as Category 4 and 5 would produce viable eggs. Categories 6 (Spawned) and 7 (atretic) characterized ovarian development that signalled the end of the spawning phase. The expected outcome was that fish assessed as Categories 6 and 7 would produce unviable eggs.

Statistical analysis of the tabulated data (Tables 5.6 – 5.8) showed that changes in the expected outcome significantly affected changes in the observed outcome, and that the year or interaction of year and expected outcome did not have an effect on the relationship of the expected and observed outcome. This meant that there was a strong relationship in the expected and observed outcome of the ultrasound imaging categories. Calculations based on the tabulation of the raw data and the error matrix showed that the accuracy of assessments was highest for Categories 1, 2, 3, 6 and 7. If fish were assessed as one of these ovarian development categories, they would not produce viable eggs. For example, Categories 1, 2 and 6 were 100% accurate based on percentage calculation of the agreement of the expected to observed outcomes for each category in each spawning trial in the raw data (Tables 5.6 – 5.8), and the probability of a correct assessment was 0.99 for Category 1, 0.92 for Category 2, and 0.87 for Category 6 based on the error matrix. The mean accuracy rate across the three years of the raw data tabulation showed that Category 4 was 32% accurate and that Category 5 was 49% accurate based on the agreement of the expected and observed outcome of viable egg production (Tables 5.7 – 5.9). The error matrix revealed that the probability of a correct assessment (i.e. the collection of viable eggs)

was 0.86 to 0.89 for Category 4 and 5, but that these two categories could produce unviable eggs and thus be confused with the outcome associated with Category 6 (Table 5.9).

The implications of the ultrasound imaging classification analysis is that ultrasound imaging assessments of ovarian development could be reliably used to decide whether or not to inject a fish with a hormone to induce spawning, especially for identifying the fish which would not produce viable eggs. These findings have direct implications for increasing the efficiency and reducing the costs associated with labor, hormones, time in commercial hatchery production. Ultrasound imaging assessments would identify fish which should not be injected with an accuracy rating close to 100% due to too early (e.g. Undeveloped, Category 1; Under-developed, Category 2) or too late (e.g. Spawned, Category 6) developmental stages of oocyte and ovarian development. These categories were therefore not suitable for use in artificial spawning. In addition, external morphology of a well-rounded abdomen that is soft to the touch by palpation - the external method of assessment in commercial hatcheries - can include fish with ovarian development and internal morphology comprised of atretic oocytes that could be identified with a 1.0 probability of a correct assessment represented by Category 7, Atretic.

Summary and Conclusions

Ultrasound images of ovarian development in adult channel catfish (3 – 5 yr old) were observed, categorized, and recorded in Baton Rouge, Louisiana, using a standardized and systematic interpretation approach. This approach included: (1) use of the initial fish handling and imaging procedures developed (Guitreau et al. 2012); (2) consistent use and reporting of control settings; (3) the recording of images during the recrudescence, spawning and regression phases of the

ovarian cycle; and (4) the use of ultrasound imaging features to distinguish ovarian morphologies (i.e. visibility, the qualitative estimation of size, and the echogenic appearance of the ovary and oocytes).

A preliminary ultrasound imaging index comprised of seven categories was developed and evaluated based on the expected and observed biological outcomes of 10 artificial spawning trials during 2008 to 2010. The first expected outcome was that fish would not produce viable eggs. This expected outcome was associated with ovarian development assessed as Categories 1 (Undeveloped), 2 (Under-developed), 3 (Developing), (6 (Spawned) and 7 (Atretic). The second expected outcome was that fish would produce viable eggs. This expected outcome was associated with ovarian development assessed as Category 4 (Advanced) and 5 (Mature). Statistical analysis of the tabulated data for the spawning trials showed that the expected outcome associated with the ultrasound imaging categories was significantly related. Based on the error matrix, fish assessed as having ovarian development Categories 1, 2, 3, 6 and 7 would very likely not produce viable eggs (0.87 to 1.0 probability), and that fish assessed as category 4 and 5 were likely to produce viable eggs (0.86 and 0.89 probability). These two predicted outcomes are directly relevant to increased efficiency in commercial hatchery production.

Correctly deciding whether or not to inject females for hormone-induced spawning is vital to efficient and successful artificial spawning, and has important economic implications on the reduction of the costs associated with labor and hormones used. Ultrasound imaging provides a direct view of the condition of ovarian development, and it may be effectively used for improving selection of females for commercial hatcheries by identifying fish which would not

produce viable eggs with 100% accuracy, and by reducing the number of fish which were injected and maximizing the number of fish which would produce viable eggs (Categories 4 and 5). The selection of fish for injection based on ultrasound imaging assessments in this study and the analysis the accuracy of these assessments provided biological insight needed for the development of a standardized ultrasound imaging ovarian reproductive index for channel catfish. At present, commercial hatcheries could procure ultrasound assessment as a service for hire, performed by trained technicians. In the future, this technology may become sufficiently defined and accepted for hatcheries to obtain equipment themselves use by hatchery personnel.

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Chapter 6
An Ultrasound Imaging Reference Guide for Channel Catfish Ovarian Development

Ultrasonography is an imaging technology used as an assistive diagnostic tool in decision-making and studies on internal anatomy such as reproductive organs in humans, livestock animals, and aquatic species (Jansen and van Os 1989; Kane et al. 2004; Medan and Abd El-Aty 2010; Novelo and Tiersch 2012). The introduction of basic grey-scale (B mode) ultrasound imaging has revolutionized studies on reproductive biology and made possible studies of internal anatomy that were otherwise not likely (Gowda et al. 2004; Ginther 2014).

Ultrasonography is defined by the use of equipment that modulates electric and sonic energy to produce frequencies higher than human hearing (usually in the range of 2 – 10 MHz for diagnostic examinations) to view images of internal anatomy (Nyland et al. 2002). The first uses of ultrasonography on humans was in the 1950s when subjects were submersed in water which conducted sound waves to generate the ultrasound images (Walsh et al. 1993). As this imaging technology advanced, ultrasound transmission gel was developed for use as a sound conductor in diagnostics in terrestrial species.

Ironically, the use of water as an ultrasound conductor was discontinued even though it was particularly suitable for species such as fish that cannot safely be held out of water for long periods of time (Walsh et al. 1993; Guitreau et al. 2012). It is because of its use in a water environment along with the rapid, real-time viewing of ovaries of channel catfish *Ictalurus*

punctatus that this technology was adapted for use in studies of fish reproduction at the Louisiana State University Agricultural Center - Aquaculture Research Station (LSUAC-ARS). This led to the development of this ultrasound imaging guide to ovarian development of channel catfish.

Before any attempt at interpretation and use of images that are illustrated in this reference guide (which took 10 years to develop since the initial studies conducted in 2004 at the LSUAC-ARS), it is important to understand that this ultrasound schematic of ovarian development of channel catfish was developed within a standardized framework.

Consistent fish handling and ultrasound imaging techniques were initially developed to optimize the use of the natural water environment of the catfish for quick, safe and minimal handling during ultrasound imaging for integration into reproduction research (Guitreau et al. 2012). Ultrasound image control settings demonstrated that different settings produced different image morphology of the same ovary in observations during hormone-induced spawning trials. One setting produced images only of the general shape of the ovary, and while another produced a view of the ovary and oocytes yielding direct biological insight into the state of the ovary through the observed morphological changes (addressed in Chapter 4 of this dissertation).

The settings selected are an essential foundation in the development of these reproductive indices and every effort was made to record and consistently apply settings during ultrasound imaging. This ensured consistency and standardization of imaging procedures, and it addressed the need

for standardized documentation and reporting of the control settings which are not typically included in publications on the use of ultrasonography in fish reproduction (Novelo and Tiersch 2012).

The guide was developed to include the biological spectrum of ovarian development during the annual reproductive cycle of the channel catfish. The images presented to illustrate each stage represent a schematic for interpreting ovarian development within an overarching framework for the full range of morphologies observed during the annual ovarian cycle. This means that actual ultrasound images will not look exactly the same as images depicted in this reference guide. The guide was developed to provide a standardized categorical viewpoint (i.e. a snapshot) of the continuous and complex ovarian development processes. The utility of this reproductive index lies in the general criteria of the echogenic morphology defined for each category that, in turn, comprises a decision-making tool to increase efficiency in reproduction for genetic improvement.

Methods

Equipment, Ultrasound Control Settings, and Fish handling. The reproductive index was developed with the use of a portable ultrasound unit with basic grey-scale imaging features – the Classic TelaVet 1000™ Veterinary Digital Ultrasound Module (Telemed UAB, Vilnius, Lithuania). The ultrasound unit was linked to a waterproof probe that produced rectangular image displays (model LV7.5/60/96) and a laptop computer for user control of ultrasound software (Echo Wave Ultrasound Software version 3.60, Telemed UAB, Vilnius, Lithuania), image storage and data analysis. Ultrasound control settings for viewing the ovary and oocytes were saved in the 'pre-set' option of the ultrasound software and were used consistently for

viewing, observing and interpreting ultrasound images of the ovaries (Table 6.1). Thirdly, consistent methods of fish handling and ultrasound imaging procedures were used for viewing and assessing adult (3-to-5-yr old) channel catfish ovaries (Guitreau et al. 2012). The fish was held in swimming position in water by a light but firm grasp of the tail immediately before the tail fin (i.e. the caudal peduncle) with one hand by the ultrasound image assessor. The probe was placed in a vertical position alongside the dorsal fin at 0 – 1 cm from the skin, along the left side of the fish with the assessor's other hand, with the tip of the probe pointing towards the bottom of the fish and the cord end of the probe pointing up (Figure 6.1).

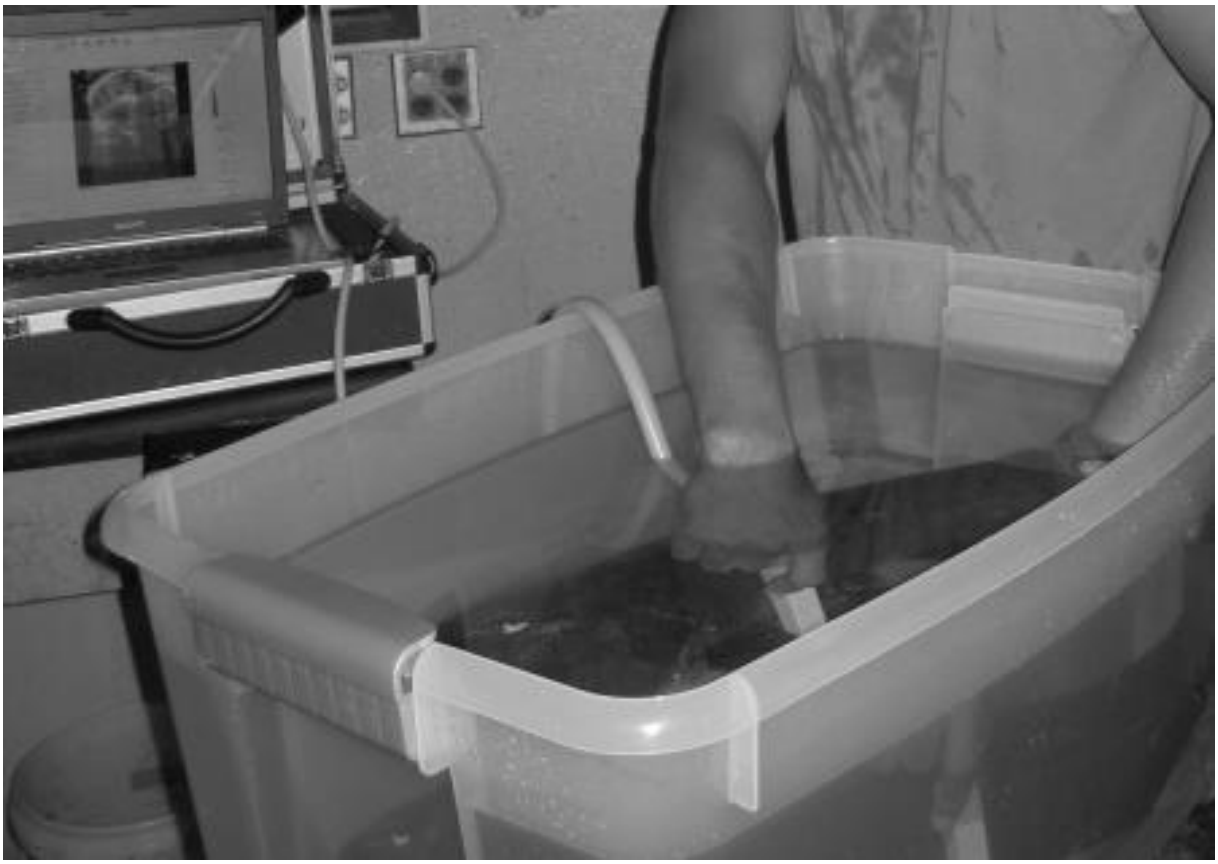


Figure 6.1 Fish was held in upright swimming position by holding the caudal peduncle with one hand and positioning the probe alongside the fish with the other for ultrasound diagnosis.

Table 6.1 Real-time B-Mode ultrasound imaging control settings used for diagnosing ovarian development of channel catfish and for the development of the standardized reproductive classification index (brief definitions from the Echo Wave Ultrasound Software Operation Manual, Revision 2.7, 2006). These settings were stored in the pre-set option of the ultrasound software to ensure consistency in use of ultrasound control settings and for quick retrieval.

Controls	Definition	Settings
Probe Frequency	Selects operating frequency from the multi-frequency probe	8 MHz
Transmit	Controls focusing at a depth (mm) location during transmission	10 mm
Depth	Sets the scanning depth	80 mm
Image Enhancement	Makes edges and border areas more visible	2
Dynamic Range	Controls image contrast	56 dB
Frame Average	Sets how many frames will be averaged to lower *noise level	4
Scroll	Shifts displayed scanning image from selected depth value	0 mm
Zoom	Sets the zoom ratio	100 %
Power	Acoustic power of ultrasound beam	90 %
Gain	Sets voltage amplitudes and echo brightness	70 %
Time-Gain Compensation (TGC)	Horizontal sliders that adjust the gain at specific depths	**TGC
Rejection	Ultrasonic signal rejection to reduce noise visibility	0 (on slider bar)

*Noise: unwanted background sound artifacts that can arise from within the scanner, the subject, the interaction of ultrasound with tissue structure, or from electronic interference from nearby equipment (Toal 1996, Hill 1989).

**TGC was adjusted along the depth settings of ultrasound penetration at 0, 20, 40, 60 and 80 mm.

The entire ovary was viewed by moving the probe towards the direction of the head and moving back towards the tail in the region between the pectoral fin and the urogenital pore, and ending in the mid-section of the ovary. Once the gross ovarian assessment was completed, the probe was placed by the dorsal fin (0 – 1 cm), the ovary was centered in the ultrasound image display, and the real-time assessment and image were recorded in video (Audio-Visual Interleave Format) and still image formats (Ultrasound Image Format, Bitmap Image Format) by control of the laptop computer by a second person under the direction of the ultrasonographer.

The ultrasound classification index presented is a schematic overview of annual ovarian development based on the standardized and systematic interpretation approach that was used to characterize and evaluate ovarian morphology to obtain biological insight (addressed in Chapter 4 of this dissertation). This index was also based on the biological insight on the ultrasound images obtained in the use and evaluation of the preliminary ultrasound imaging index (addressed in Chapter 5 of this dissertation).

The ultrasound image selected to illustrate each ultrasound category in the index was presented along with the histological image of the same ovary. The ovaries used to create the histological images were preserved at the same time the ultrasound images were recorded. The method used to create the histological images was described in Appendix C of this dissertation (Novelo et al. 2011). The histology images in this reference guide were included to provide a microscopic viewpoint of the gross morphology observed by use of ultrasonography.

Finally, some basic knowledge of the annual cycle of channel catfish is essential for the reader to use of this reference guide, especially because the foundation of the categories developed was based on biological inferences made from observations of ovarian ultrasound images throughout the annual cycle.

Results

Adult channel catfish undergo an annual ovarian cycle comprised of four phases: 1) recruitment of oogonia, 2) growth and development of ovary and oocytes (recrudescence), 3) spawning, and 4) resorption (atresia) (Silverstein and Small 2004). A standardized ultrasound imaging classification index was created based on the ovarian cycle.

A fundamental component for understanding and interpreting the images produced was identifying the relationship of the position of the probe on external anatomy, and the cross-sectional structures in the ultrasound image display (Figure 6.2). This relationship of the fish handling and ultrasound imaging procedures, i.e. fish orientation and probe positioning during scanning, should be recognized to identify the position of the probe and the anatomical structures in the image (Figure 6.2).

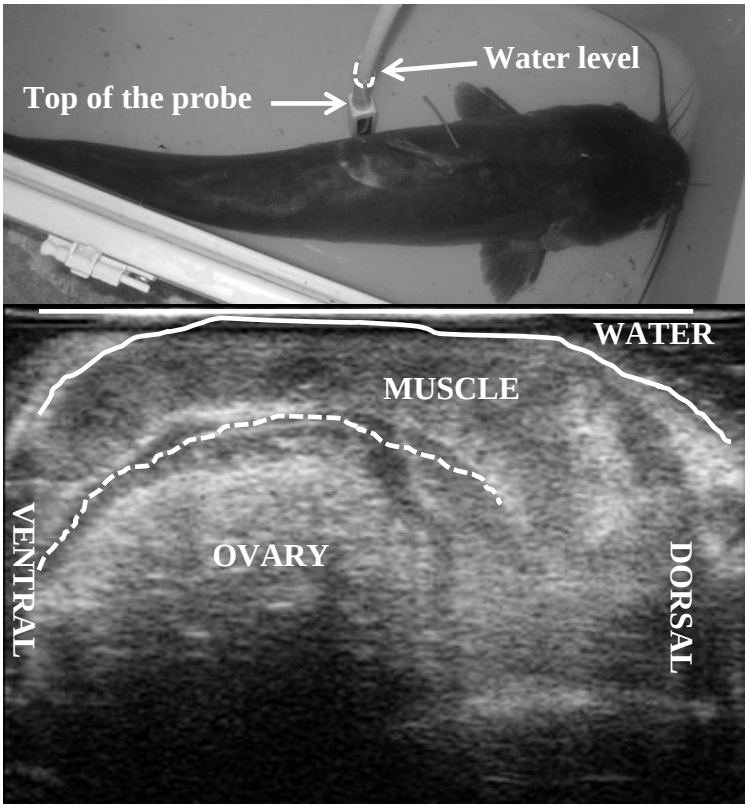


Figure 6.2 The position of the probe (indicated by the dashed and solid white lines) was at the top of the image immediately next to the skin (curved line), with the top of the fish (in the area of the dorsal fin) on the right side of the image, and the bottom of the fish (the abdomen area) on the left side of the image. The ovary appeared as if the fish were lying on its side.

Knowledge of the occurrence of these ultrasound images during the ovarian cycle was another fundamental aspect for understanding and interpreting images of ovarian development.

The ultrasound images were interpreted and classified in terms of the visibility, relative size and appearance of the ovary and oocytes (Table 6.2, 6.3). These categories were developed to represent the recrudescence, spawning and resorption phases of the ovarian cycle (Table 6.2, 6.3).

Images of ovarian development represented by Category 1 (Undeveloped) were observed in highest occurrence during early recrudescence and slow growth during October and November in the Fall. Images of ovarian development represented by Category 2 (Under-developed) and 3 (Developing) were in highest occurrence during February and March.

Images of ovarian development representative of Categories 4 (Advanced), 5 (Mature), 6 (Spawned), and 7 (Atretic) were observed in highest occurrence during the spawning season, with a higher incidence of occurrence of Categories 6 and 7 towards the end of the spawning season.

The classification index was also illustrated by use of seven ultrasound images to serve as visual illustrations with a corresponding histological image. This was followed by a detailed description of the visibility, relative size and appearance of the ovary and oocytes in the ultrasound images and observed features in the histology (Figures 6.3 – 6.9).

Finally, ultrasound images and condensed textual descriptions are presented as a unified classification key for quick reference to all categories (Table 6.4).

Table 6.2 Textual descriptions of the visibility, relative size and echogenic appearance of ovaries for 7 ultrasound imaging categories representative of ovarian development during the channel catfish ovarian cycle.

Category	Ovary: Visibility/Size	Appearance
1. Undeveloped	Visible, small	Homogenous greyish mass
2. Under-developed	Distinctly visible, small	First appearance of heterogeneous pattern
3. Developing	Highly discernible, visibly enlarged	Increased heterogeneity
4. Advanced	Enlarged, closer proximity to the skin	Complex organized heterogenous structure
5. Mature	Largest size, closest proximity to skin, dominates image	Complex, highly organized heterogenous structure
6. Spawned	Small, difficult to distinguish from adjacent tissue	Similar echogenic texture as surrounding tissue
7. Atretic	Varied size from large to small, distinct and visible	Irregular internal echogenic texture

Table 6.3 Textual descriptions of the visibility, relative size and echogenic appearance of oocytes for 7 ultrasound imaging categories representative of ovarian development during the channel catfish ovarian cycle.

Category	Oocyte: Visibility/Size	Appearance
1. Undeveloped	No oocytes visible	None
2. Under-developed	Visible, very small, no distinct individual shape	Whitish specked granules against darker background
3. Developing	Increased visibility, small, compact cohort appearance in the ovary	Beginning of discernible shape, expanding brighter structure in a greyish dark background
4. Advanced	Individually distinguished	Bright enlarging center and dark peripheral structure
5. Mature	Highly visible individual and enlarged oocytes	Individual shape, generally oval, comprised of a bright center and dark periphery
6. Spawned	No oocytes likely visible	If visible, devoid of structure and shape, amorphous
7. Atretic	Visible but irregular in appearance	Amorphous with visual appearance of degeneration

The Ultrasound Image Profile of Channel Catfish Ovarian Development with Corresponding Histological Images (Figures 6.3 – 6.9).

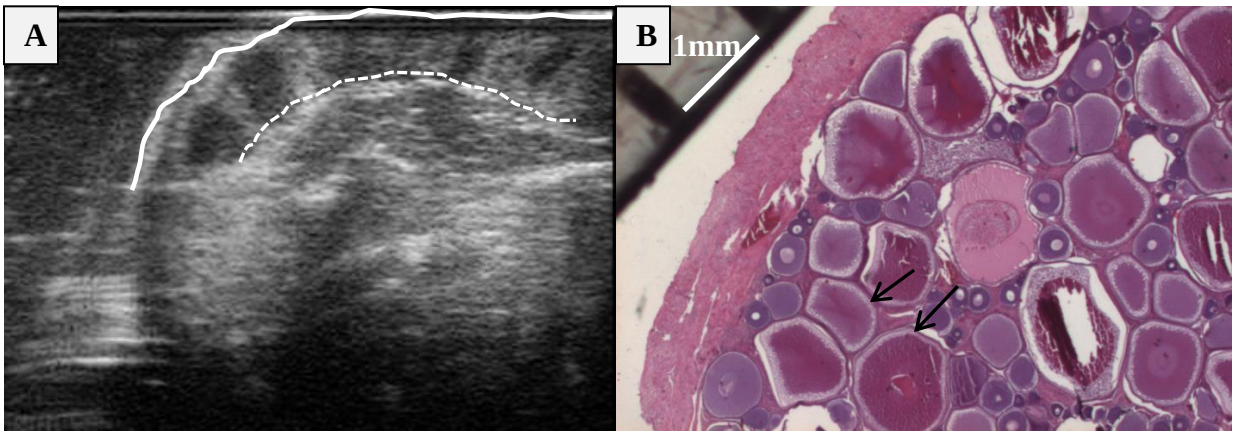


Figure 6.3 Category 1. Undeveloped. The ultrasound image (A) represented early and slow growth during the recrudescence phase. The corresponding histological profile (B) of the same ovary in the ultrasound image revealed small oocytes generally less than 1mm (ruler black bar) characterized by vacuoles (arrow) which do not stain with hematoxylin or eosin appearing along the periphery of the oocyte (Grizzle 1985). Vacuolated oocytes may range from 240 – 650 mm. In addition, oogonia and previtellogenic oocytes which may range from 12 to 120 mm (Grizzle 1985) were observed.

Ultrasound Category 1, Undeveloped. The ovaries were visible, and identified by the circular shape of its outer wall (dashed line) which appears at its furthest distance from the skin (white line) compared to the other categories developed for the recrudescence phase (i.e. categories 2, 3, 4, and 5). The ovaries appear small, but may vary in size. The key echogenic feature for this category is the homogenous greyish internal appearance of the ovary. No oocyte shape or structure is visible and they are expressed in the ultrasound image within the greyish internal appearance of the ovary. This type of image was observed with high occurrence during October and November during the early and slow growth period in Louisiana.

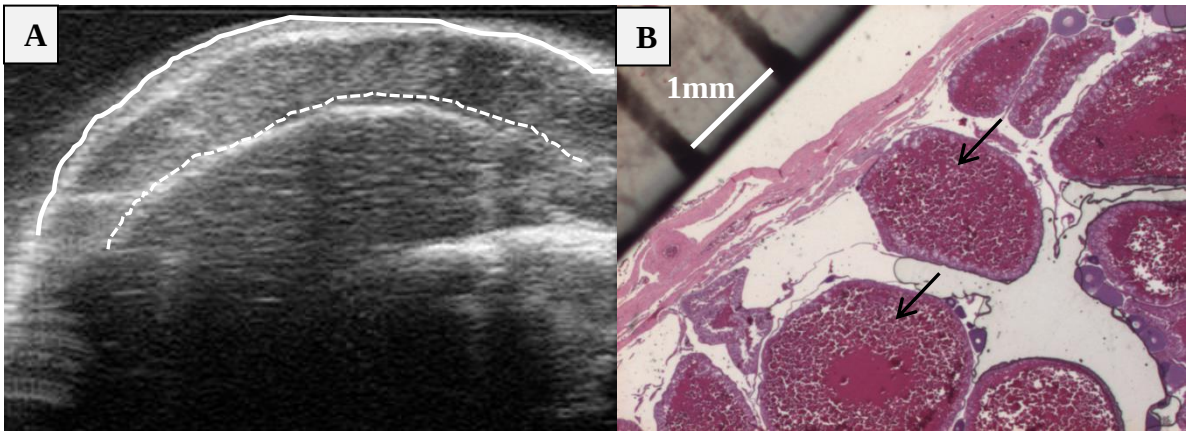


Figure 6.4 Category 2. Under-developed. The ultrasound image (A) represented the first visible sign of vitellogenic activity and occurred early during the recrudescence phase. The corresponding histological profile image (B) of the same ovary in the ultrasound image revealed oocytes characterized by yolk granules (arrow) which filled most of the oocyte cytoplasm.

Ultrasound Category 2, Under-developed. The ovaries were visible, with a clear delineation of ovarian wall closest to skin, which appears distant to the skin (white line); the entire ovarian wall surrounding the expanding ovary begins to be discernible. Ovaries appear to extend more dorsally and ventrally (towards the top and bottom of the fish) than laterally (towards the skin). The key echogenic feature for this category was the appearance of the first visible heterogeneous texture within the ovarian cross-section. This is caused by the growing oocytes that were barely visible, with no unique shape or form, but which they are expressed in the ultrasound image as grainy and specked texture in a darker background within the ovary, which coincides with more lipid content from vitellogenic activity. This type of ultrasound image was observed with high occurrence during February and March during the recrudescence period in Louisiana.

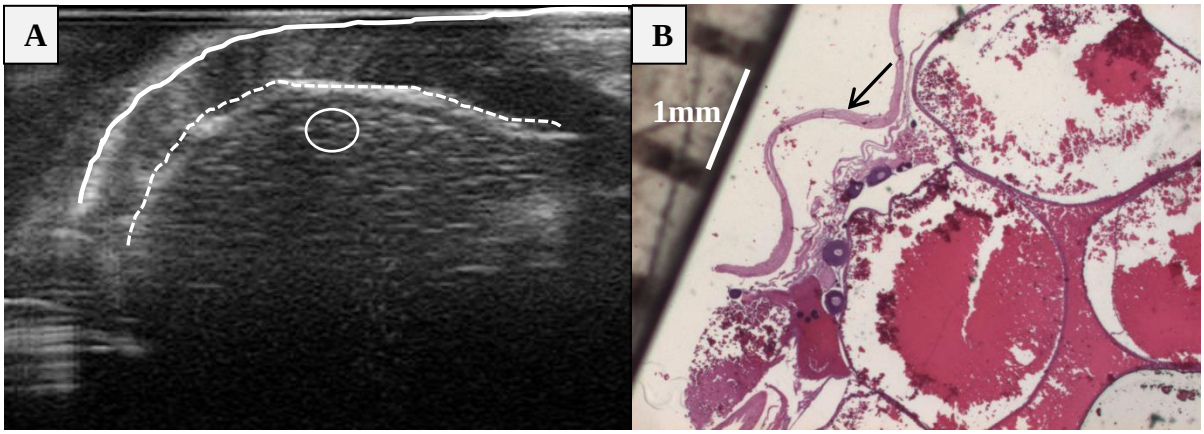


Figure 6.5 Category 3. Developing. The ultrasound image (A) represented the beginning of fast growth period during the recrudescence phase. The corresponding histological profile (B) of the same ovary in the ultrasound image was observed with high occurrence during February and March during the recrudescence period in Louisiana. The histological profile revealed larger oocytes with and a thin ovarian wall (arrow).

Ultrasound Category 3, Developing. Ovaries were highly discernible, with a clear delineation of ovarian wall which appeared closer to the skin (white line). The ovary was visibly enlarging with expansion noticeable throughout the ventral, dorsal and lateral (from top to bottom and towards the skin) cross-section of the ovary. The characteristic feature for ovaries in this category was the increased heterogeneous texture (white oval) of the area within the ovarian cross-section. This was caused by the visible growth of the oocytes that were beginning to acquire a visible shape and was expressed in the ultrasound image as a light grey to white texture of the individual oocyte structures which together comprised a compact cohort appearance within the ovary. Although oocytes begin to acquire higher visibility their individual ultrasound image structures were still developing (white oval shape) and the oocytes appeared small and difficult to discern individually. This type of image was observed with higher occurrence in February and March in Louisiana.

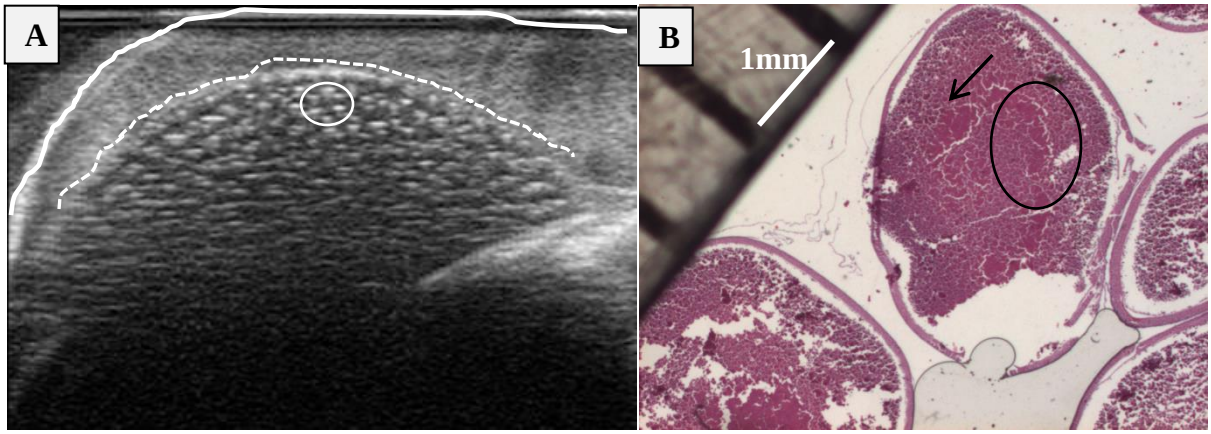


Figure 6.6 Category 4. Advanced. The ultrasound image (A) represented the fast growth period and advance development during recrudescence. The histological profile (B) of the same ovary in the ultrasound image revealed large oocytes in late vitelline stage; although distinct yolk granules were observed (arrow), the yolk granules were in the process of coallesence (oval shape within the cytoplasm).

Ultrasound Category 4, Advanced. Ovaries were highly visible, prominently expanded. The characteristic feature for ovaries in this category was the organized structured heterogeneity within the ovarian cross-section. This was caused by highly visible oocytes which had two recognizable structures – a whitish center and a darker periphery – which identified individual oocytes. For example, six individual oocyte structures may be counted by first identifying the bright whitish centers within the oval shape (white-lined oval) illustrated in the image. A clear oocyte organization is apparent, contributing to a complex and clearer image of the ovary and the oocytes. This type of image was observed with higher occurrence before and in the early part of the natural spawning season in April and May in Louisiana. The corresponding histological profile of the ultrasound image was observed with high occurrence during February and March during the recrudescence period in Louisiana.

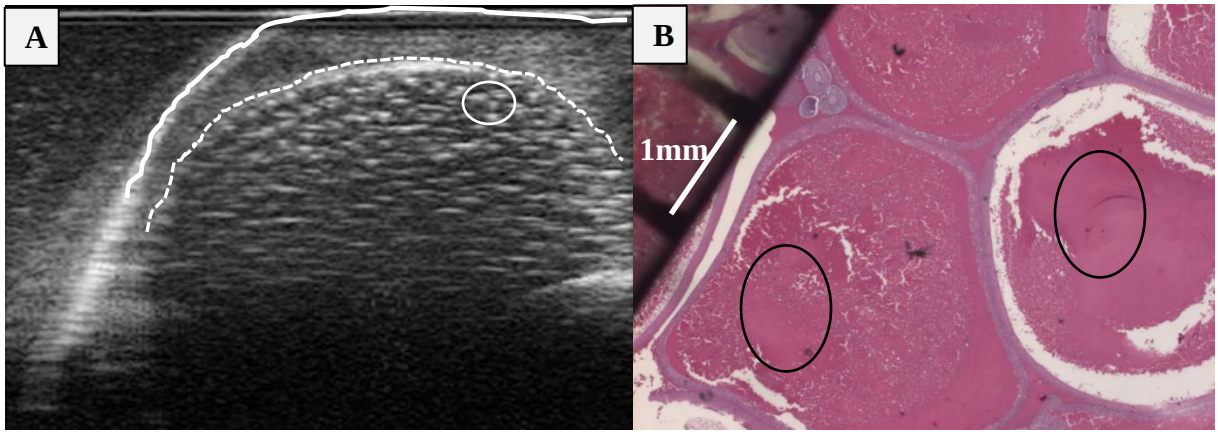


Figure 6.7 Category 5. Mature. The ultrasound image (A) represented transition to the spawning period towards the end of the vitellogenesis. The corresponding histological profile (B) of the same ovary in the ultrasound image revealed large oocytes in late vitelline stage that contained coalesced yolk (ovals within the cytoplasm).

Ultrasound Category 5, Mature. Ovaries and oocytes appeared large and prominent in the image, and the features of the ovary and oocytes could be highly discerned. The characteristic feature of the ovaries was the highly organized heterogenous texture within the cross-sectional area which displayed the individual bright white and darker peripheral structures comprising individual oocytes (e.g. four oocyte structures in the white oval). This type of image was observed during the natural spawning season in Louisiana.

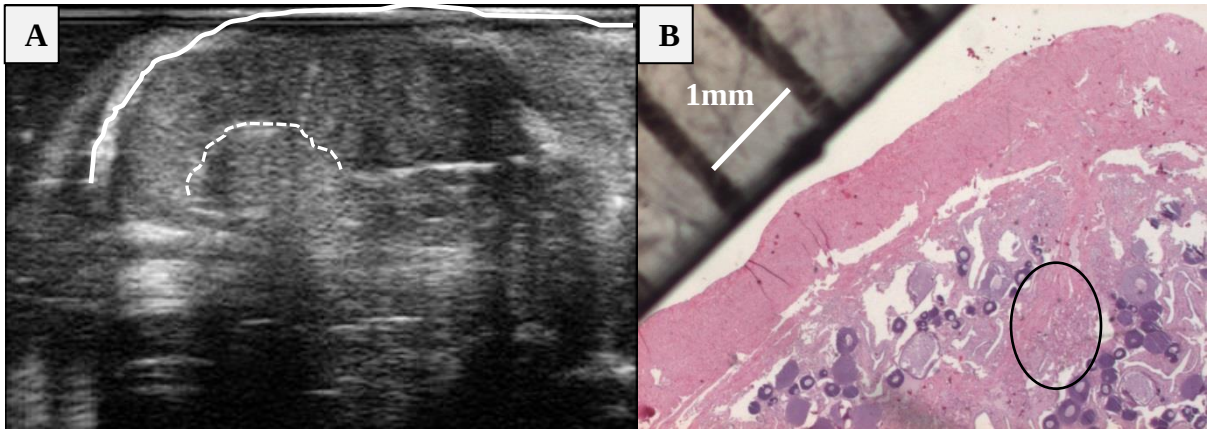


Figure 6.8 Spawned. The ultrasound image (A) represented the post spawn or 'spent' state of the ovary in an advanced regressed state. The corresponding histological profile (B) of the same ovary in the ultrasound image revealed a thickened ovarian wall, remnants of atretic oocytes (oval) and oogonia.

Ultrasound Category 6, Spawned. The ovaries were very small and difficult to identify. The predominant feature of ovaries in this category was that they were difficult to distinguish as they appeared small and similar in texture to the surrounding tissues. No oocytes were likely visible, but if they were they were few appeared deformed. This type of image was observed during the natural spawning season in Louisiana.

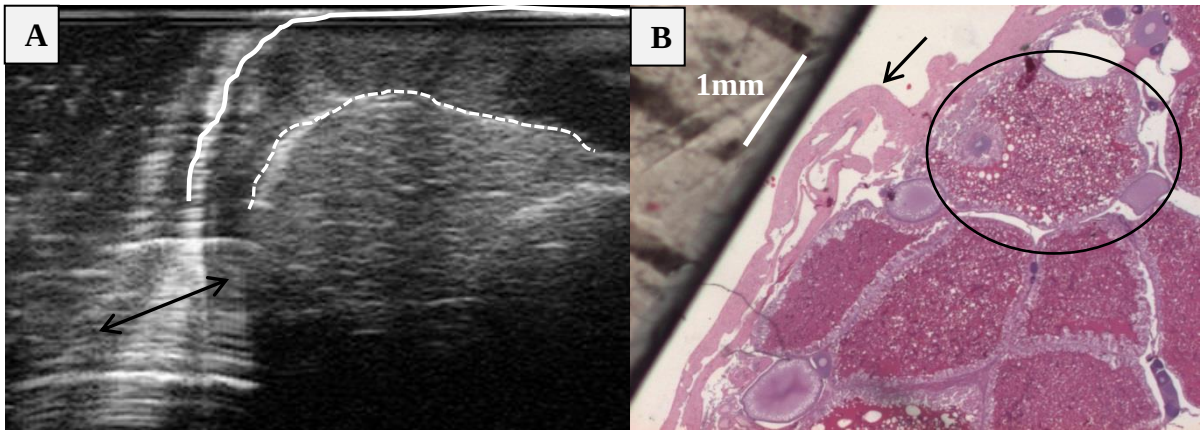


Figure 6.9 Category 7. Atretic. The ultrasound image (A) represented visible regressing processes. The corresponding histological profile (B) of the same ovary in the ultrasound image revealed irregular-shaped oocytes (oval) with a thickening ovarian wall (arrow).

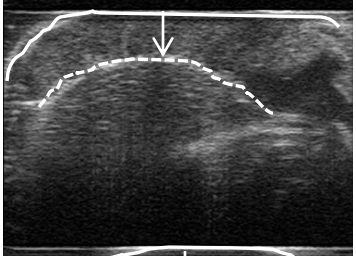
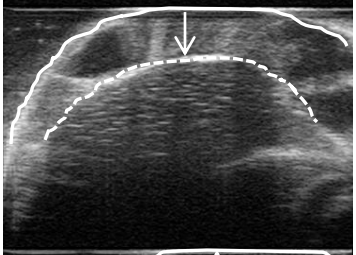
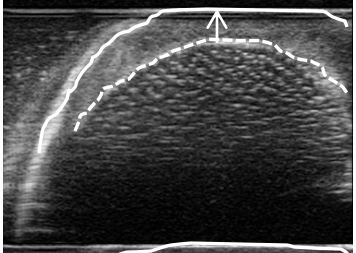
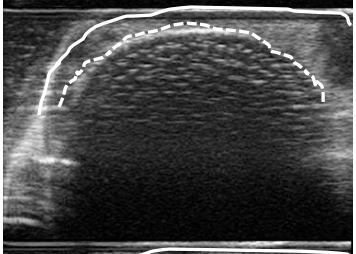
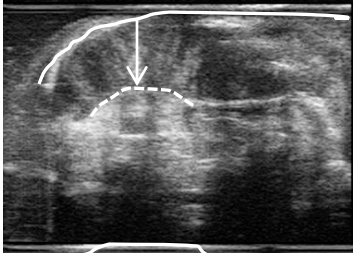
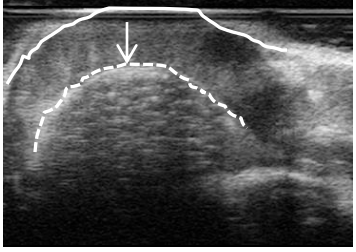
Ultrasound Category 7, Atretic. The double-headed black arrow indicates the ultrasound image artefact generated by the movement of the fish recording of the ultrasound image. The predominant feature of ovaries and oocytes in this category was that they were highly variable shapes and sizes that lacked structural integrity and uniformity and appeared irregular. Ovaries were distinct and ranged from large to small, from rounded to unstructured, with irregular and unorganized internal texture appearance. Similarly, oocytes were irregular amorphous shapes with the visual appearance of degeneration. This type of image was observed with high occurrence late during the natural spawning season in Louisiana.

Discussion

The use of ultrasound imaging in channel catfish reproduction provided the opportunity to create a standardized platform for obtaining new insights and for performing studies based on a visual, qualitative template for discriminating images of ovarian development anytime during the annual reproductive cycle. In addition, it is a tool for use in the effort to maximize reproductive efficiency, in particular by identifying females that are not ready for hormone-induced spawning and enabling focus on females with a higher chance of spawning.

Considerations for Use of this Reproductive Index. This classification index for the channel catfish ovary was developed as an aid to understanding, interpreting and assessing ovarian reproductive development using ultrasonography. However, an understanding of the biological phases and timing of development of channel catfish at different locations (i.e. latitudes) is essential to its use, as the images were associated with different developmental reproductive states during the ovarian annual cycle, which is entrained by temperature (Silverstein and Small 2004). In Kentucky, for instance, channel catfish spawn 3 to 6 weeks later than in more southern states such as Louisiana (ksuaquaculture.org/Species/Catfish.htm). In Southern Louisiana the recrudescence period has been reported to start in October, and the natural spawning season starts after water temperatures stabilize at 21 C (70 F) and may start as early as mid-April and last up to mid-July (Jensen 1988; Banks et al. 1999). Therefore, too interpret ultrasound images, this guide should be used in the context of the phases of the ovarian cycle, the temperature profile, and the associated biological state of the fish at a particular location.

Table 6.4 Ultrasonography classification for ovarian development in adult channel catfish

	Category 1. Undeveloped. Represents early and slow growth. Ovaries appear as a small, homogenous grey mass, with a distinct curved shape (dashed line) distant from the skin (arrow). No oocytes visible.
	Category 2. Under-developed. Represents visible sign of vitellogenic activity. Ovaries visible, small, extending more dorsally and ventrally than towards skin, distant from skin (solid line), heterogeneous pattern introduced from first visible sign of oocytes. Oocytes appear as small grainy whitish speckles in darker grey background in ovary.
	Category 3. Developing. Represents the beginning of the fast growth period. Ovaries highly visible, enlarging, with expansion noticeable throughout (ventral, dorsal, towards skin), distant from skin, increased heterogeneity. Oocytes begin to acquire visible shape as small structures with light grey to white texture and compact appearance.
	Category 4. Advanced. Represents the fast growth period, advanced development late in the recrudescence phase. Ovaries are prominently expanded, closer to the skin, complex organized heterogenous structures. Oocytes highly visible, individually distinguishable with a bright enlarged center and dark periphery.
	Category 5. Mature. Represents transition from the recrudescence period to the spawning period. Ovaries and oocytes distinct. Ovaries are large, in close proximity to the skin, highly organized and complex structure. Oocytes structure highly defined, comprised of bright center and dark ring-like periphery, individual shapes visible.
	Category 6. Spawned. Represents post-spawn (spent); i.e. after the spawning period. Ovaries very small, barely visible, difficult to distinguish and identify from surrounding tissues. Oocytes likely not visible; if visible, appear as amorphous shape.
	Category 7. Atretic. The images represent the period at the end of the spawning phase. Ovaries and oocytes are irregular in size and shape. Ovaries visible, distinct, range from large to small, from rounded to unstructured, with irregular internal texture. Oocytes usually visible, with amorphous appearance of degeneration.

Secondly, a classification key was developed as an aid for assessment as a visual and textual illustration (Table 6.4). This classification key may be used as a quick reference guide to interpret ultrasound images of ovarian development. Thirdly, the use of basic grey-scale real-time B-Mode ultrasound imaging equipment was used to develop this reproductive index. Ultrasound units may vary in price from less than $10,000 (for units similar to the equipment used for this reproductive index) to more than $100, 000 for units used in human medicine (Novelo and Tiersch 2012). They vary in the type of user controls (e.g. physical sliders and knobs; software sliders and buttons) and the names given to these controls depending on the model (Nyland et al. 2002). However, assessments of the channel catfish ovary can be done using basic grey-scale features described for the equipment in the Methods section, which may cost approximately $10,000 or less (refurbished models) (Novelo and Tiersch 2012). Although ultrasound units may be inconsistent in the use of names for controls, the functions are similar and they will have basic shared features such as the frequency used, power, gain, ultrasound penetration depth, and focus depth. Potential users should familiarize themselves with the equipment, the user interface (software or physical knobs), the control settings, and the function of each of the controls, and use the same or similar settings as reported in this guide. Once the control settings are adjusted to the same or similar to the ones reported in this guide, record them so that it is the same setting used for all ultrasound imaging assessments and for studies to be replicated and compared.

Summary and Conclusions

This guide was developed to provide a standard platform for ovarian assessment in research and commercial scale activities based on recommended imaging and handling procedures. A seven-category ultrasound imaging index was developed to represent ovarian and oocyte morphology

during recrudescence, spawning and resorption phases. This ultrasonographic approach to assessment provides direct visual evaluation of biological processes that enable further studies of ovarian morphology that would be useful for genetic improvement such for commercial production of the hybrid cross of the channel catfish female x blue catfish *Ictalurus furcatus* male. Therefore, this ultrasonographic method of assessment provides a practical method for assisting genetic improvement in research and commercial production.

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Chapter 7
Ultrasound Imaging Reproductive Assessment of Channel Catfish Ovaries in Commercial-Scale Hormone-Induced Spawning

Commercial catfish production in the US has historically referred to the exclusive culture of channel catfish *Ictalurus punctatus*, the leading foodfish industry in US aquaculture. Its production yielded total aquaculture sales exceeding $400 million in 2010 but declined to $342 in 2013 (USDA 1988-2013). While the channel catfish remains the primary species in the US catfish industry, the F_1 hybrid from the artificial breeding of the female channel catfish × male blue catfish *I. furcatus* (C × B hybrids) has increasingly been cultured. The first reported commercial-scale production of C × B hybrids was in 2001 by one private hatchery in Mississippi, which produced 2 million fry a season (Chatakondi 2012). In January 2003, C × B hybrids were raised in 2.1% of all food size-fish surface acres, corresponding to 1.2% of the total food size-fish inventory, while in January 2010 C × B hybrids were raised in 21.2% of the food size-fish operations, corresponding to 5.9% of the total food size fish inventory (USDA 1988-2013). C × B hybrid fry production increased to 111 million by six catfish hatcheries in Mississippi and Arkansas in 2011, and production is expected to exceed 200 million fry in 2014 (Chatakondi 2012).

Although early studies of channel and blue catfish pond spawning resulted in poor rates of natural spawning due to behavioral inhibitions (Perry 1973; Tave and Smitherman 1982; Wolters and Tiersch 2004), interspecies hybridization demonstrated benefits such as increased growth rate, processing yield, and disease resistance (Giudice 1966; Argue et al. 2003; Li et al. 2004; Arias et al. 2012). On-going research on C × B hybrids addresses different aspects of aquaculture, including fingerling prices and profitability (Ligeon et al. 2004; Kumar and Engle

2011), use of cryopreserved sperm in artificial reproduction and commercial application (Hu et al. 2014), and induced spawning for collection of eggs (Phelps et al. 2007; Kristanto et al. 2009; Phelps et al. 2011). Meanwhile, intraspecies studies have worked towards genetic improvement of commercially desirable traits such as growth and processing (Dunham et al. 1983), disease resistance and stress (Wolters et al. 1996; Bosworth et al. 2004; Peterson et al. 2010), reproductive performance (Broussard Jr and Stickney 1981; Dunham et al. 1983), and artificial fertilization with cryopreserved sperm (Cuevas-Uribe et al. 2011).

Whether an intraspecies or interspecies approach is taken in research or commercial production, increasing control over parentage selection to secure seedstock is intrinsic to genetic improvement and successful aquaculture (Wolters and Tiersch 2004). In particular, selection of channel catfish females for use in commercial egg production and fertilization is a critical component of induced spawning and fertilization because it has a direct effect on the developmental ability of the eggs produced as a result of ovarian stage at the time of induced spawning (Bobe and Labbé 2010).

The technique used for selection of channel catfish females is specific to farms, dependent on the experience and criteria of farm workers which assess the females, and few studies have addressed the commercial-scale selection of females for production of eggs in commercial spawning (Phelps et al. 2011). Amongst the techniques available for selection of females, the most commonly used commercial-scale technique is the visual inspection of the external morphology of the females (Lee 1991). The basic inspection requirements are that the ovary is large such that the abdominal protrusion appears swollen, rounded, and that the genital papilla is

slightly raised and reddish in color (Lee 1991; Phelps et al. 2011). By these criteria, any fish that does not have a 'well-rounded' abdomen with a swollen red papilla may be rejected, including fish with a flat abdominal area. This technique is typically in use in commercial-scale enterprises because it provides a fast method of assessing fish by inspecting external morphology and relating this to the reproductive readiness for artificial spawning.

In contrast to external inspection for assessing ovarian reproductive development, the use of ultrasound imaging – which utilizes acoustic and electrical energy to display internal anatomy – provides real-time visual insight of internal ovarian morphology using minimal fish handling techniques (Novelo et al. 2011; Guitreau et al. 2012). Because ovarian morphology can be easily and directly viewed, this imaging technology demonstrates a strong potential for assessing channel catfish reproductive condition for commercial applications based on initial handling and imaging procedures developed (Guitreau et al. 2012). At a procedural and technical level, ultrasonography in channel catfish was shown to rapidly (less than 1 min per fish) provide clearly defined ovarian morphologies that could be systematically interpreted and organized to represent the development process during the ovarian cycle (Chapters 4, 5 and 6 of this dissertation).

This use in channel catfish reproduction provided a decision-making tool with potentially great utility in assessing commercially relevant numbers of catfish which would eliminate those with ovarian morphologies not associated with viable egg production (Chapter 5 and 6 of this dissertation). For example, ovaries assessed as Category 1 (Undeveloped), Category 2 (Under-developed) and Category 3 (Developing) were representative of slow growth during the

recrudescence phase and not ready for hormone-induced spawning, and Category 7 (Atretic) ovaries could be incorrectly assessed by external morphology as a fish that is ready to spawn if the abdomen appears large and soft.

The elimination of fish by ultrasound that would not produce viable eggs yet would have passed external inspection (e.g. Category 7, Atretic), and the selection of ovarian morphologies by ultrasound that are associated with viable egg production (e.g. Categories 4, Advanced and 5, Mature) would improve efficiency of reproduction. This in turn would contribute to savings associated with costs of hormone use, time and labor involved in the selection of fish for artificial spawning and the production of viable eggs at a commercial-scale.

The goal of this study was to use the ultrasound classification index of channel catfish ovarian development that was being developed (Chapter 5 in this dissertation) as a decision-making tool in the production of C × B hybrids. The objectives were to use the classification index to: (1) compare ovarian development of adult channel catfish conditioned for reproduction at ambient temperatures in Louisiana and Arkansas; (2) decide which fish conditioned for reproduction in Louisiana should be transported for artificial spawning and commercial hatchery production of C × B hybrids in Arkansas, and (3) compare the quality of eggs related to the ultrasound imaging assessments of fish conditioned in Louisiana and in Arkansas that were selected for commercial hatchery production in Arkansas.

Methods

Broodstock and Location. Broodstock channel catfish (3,191 kg; approximately 1,400 fish) were transported from Baxter Lands Company (BLC) hatchery in Arkansas City, Arkansas

(Latitude 33° 36’ 32” N, Longitude 91° 12’ 13” W) to the Louisiana State University Agriculture Center Aquaculture Research Station (LSUAC-ARS) in Baton Rouge, Louisiana (Latitude 30° 32’ 24” N, 91° 5’ 24” W) on November 7, 2007. These catfish were stocked into two ponds (0.30 ha/pond) at the LSUAC-ARS to be overwintered and conditioned for reproduction at ambient temperatures. The fish were fed a commercial catfish diet (Aquaxcel, Cargill™, 45% protein) at 1 to 3% of body weight 2 to 3 times/week.

Temperature. Temperature dataloggers (Model SK100 Dickson Compact Temperature Datalogger, Addison, Illinois) were placed in each pond to record ambient water temperature from November 2007 at the time of arrival until April and May 2008 at the time of ultrasound imaging assessments for identifying fish for spawning at the BLC hatchery. Degree-day profiles were calculated for channel catfish broodstock ponds in Louisiana and in Arkansas as the sum of the daily difference between the mean daily temperature and 21 C, the recommended threshold temperature (Pawiroredjo et al. 2008). This thermal profile was compared to standardized degree-day guidelines developed for the onset of spawning (10% spawning, at 57 – 81 degree-days), the median (50% spawning at 99 – 129 degree-days) and the conclusion of spawning (90% spawning at 150 to 172 degree-days) (Pawiroredjo et al. 2008).

Ultrasound Imaging Equipment and Settings. The ultrasound imaging equipment used for assessment and recording images of channel catfish ovaries was a portable ultrasound unit, the Classic TelaVet 1000™ Veterinary Digital Ultrasound Module (Telemed UAB, Vilnius, Lithuania), and a linear probe (model LV7.5/60/96). The same ultrasound control settings were used for ovarian development of channel catfish sampled at the LSUAC-ARS as at the BLC

hatchery (Table 7. 1). The images were recorded in ultrasound image format (USI) and Windows Bitmap Image format (BMP).

Fish Handling and Ultrasound Imaging Procedures. The fish captured by seine from broodstock holding ponds at the LSUAC-ARS were placed into a 2800-L water volume fish hauler. The fish hauler was provided with compressed oxygen and transported to concrete flow-through raceways of 1900-L volume into which running water from an ambient temperature pond was pumped. Each raceway held 50 to 60 fish. Once the fish were in the raceways, they were captured and moved using dip nets and fish baskets one at a time for ultrasound imaging.

Each fish was placed in a portable 49-L cooler (Sportsman™ 52 Quart, Igloo Products Corp., Katy, TX, USA) which was half filled with water (20 – 25 L, sufficient to submerge the fish). The fish were lightly held by the caudal peduncle in an upright swimming position. The ultrasound imaging probe (transducer) was placed alongside the dorsal fin. The ovaries were viewed and assessed by moving the probe alongside the lateral aspect of the fish in swimming position towards the anterior and the posterior of the ovary as displayed in the ultrasound imaging monitor, in the area between the pectoral and the pelvic fin towards the urogenital pore. This provided a general scan to quickly view the entire ovary for assessment of development. This procedure was repeated until a diagnosis was made. Finally, the probe was held in one position alongside the dorsal fin, and once the image of the ovarian cross-section was centered, and the skin of the fish was within 0 to 1 cm of the probe in the ultrasound image display, the assessment and image were recorded. The fish was immediately removed from the cooler after which the next fish underwent the same procedure.

Table 7.1 Scanner controls in B-Mode used to assess real-time cross-sectional views of the channel catfish ovary at a commercial-scale were listed below (brief definitions from the Echo Wave Ultrasound Software Operation Manual, Revision 2.7, 2006). These control settings were stored in the pre-set option for ensuring consistency in use of ultrasound controls and for quick retrieval.

Controls	Brief Definition	Setting
Probe Frequency	Selects operating frequency from the multi-frequency probe	8 MHz
Transmit	Controls focusing at a depth (mm) location during transmission	10 mm
Depth	Sets the scanning depth	80 mm
Image Enhancement	Makes edges and border areas more visible	2
Dynamic Range	Controls image contrast	56 dB
Frame Average	Sets how many frames will be averaged to lower *noise level	4
Zoom	Sets the zoom ratio	100%
Power	Acoustic power of ultrasound beam	90%
Gain	Sets voltage amplitudes and echo brightness	70%
Time-Gain Compensation (TGC)	Horizontal sliders that adjust the gain at specific depths	**TGC
Rejection	Ultrasonic signal rejection to reduce noise visibility	0
Image format Recorded	Image format selected for recording ultrasound images of the ovaries	BMP, USI

*Noise: unwanted background sound artifacts that can arise from within the scanner, the subject, the interaction of ultrasound with tissue structure, or from electronic interference from nearby equipment (Toal 1996, Hill 1989).
**TGC was adjusted along the depth settings of ultrasound penetration at 0, 20, 40, 60 and 80 mm.

The same fish handling and imaging procedures were used at BLC hatchery in Arkansas, except that fish were assessed beside of the pond, and an electric generator was used to power the ultrasound unit.

Assessments of Ovarian Development. Ovarian development of channel catfish was assessed using the ultrasound classification index developed at the LSUAC-ARS (Chapters 5 and 6 in this dissertation). This index was comprised of seven categories defined by

ovary and oocyte visibility, size, and echogenic appearance (the brightness and grey-scale texture and structure appearance resulting from the interaction of ultrasound with the ovarian tissues) (Table 7.2, 7.3).

Table 7.2 Ultrasound imaging assessments were based on the visibility, size and echogenic appearance of the ovary along with the oocyte morphology described in Table 7.3, which together defined the seven categories comprising the ultrasound imaging reproductive index.

Category	Ovary: Visibility/Size	Appearance
1. Undeveloped	Visible, small	Homogenous greyish mass
2. Under-developed	Visually distinct, small	Appearance of heterogeneous pattern
3. Developing	Highly discernible, enlarging	Increased heterogeneity
4. Advanced	Enlarged, closer to the skin	Complex heterogenous structure
5. Mature	Largest size, closer to the skin, dominates image	Complex, highly organized heterogenous structure
6. Spawned	Very small, difficult to distinguish from other tissues	Similar echogenic texture as surrounding tissue
7. Atretic	Visually distinct, size varies from small to large	Irregular internal echogenic texture

Table 7.3 Ultrasound imaging assessments were based on the visibility, size and echogenic appearance of the oocytes along with the morphology of the ovary described in Table 7.2, which together defined the seven categories comprising the ultrasound imaging reproductive index.

Category	Oocyte: Visibility/Size	Appearance
1. Undeveloped	No oocytes visible	None
2. Under-developed	Visible, very small, no distinct individual shape	Whitish specked granules against darker background
3. Developing	Higher visibility, small, compact cohort appearance	Beginning of discernible shape, expanding brighter structure in a greyish dark background
4. Advanced	Individually distinguished in the ovary	Bright enlarging center and dark peripheral structure
5. Mature	Highly visible individual and enlarged oocytes	Individual shape, generally oval, comprised of a bright center and dark periphery
6. Spawned	No oocytes likely visible	If visible devoid of structure and shape, amorphous
7. Atretic	visible but irregular in appearance	amorphous with visual appearance of degeneration

Categories 1, 2, and 3 represented ovarian development observed during the recrudescence phase, and Categories 4 and 5 represented ovarian development associated with the production of viable eggs (Table 7.2, 7.3). Categories 6 and 7 represented ovarian development observed during the resorption phase.

Intermediate ultrasound imaging assessment categories (such as "3.5" and "4.5") were used in this study, which coincided with the first year of the preliminary development phase of the reproductive index (Chapter 5 in this dissertation). This exploratory intermediate categorical assessment was discontinued to develop an assessment that was based on a general categorical index based on the ovarian cycle (Chapter 6 in this dissertation). Therefore, an equivalency table was designed for standardization of ultrasonography assessment data, which were reported in Appendix B Section 4 (Table 7.4).

Table 7.4 An equivalency table was developed for standardization of raw data collected during the preliminary development of the ultrasound imaging reproductive index for use in assessment of ovarian development in channel catfish.

Category	Equivalency
1. Undeveloped	1. Undeveloped
2. Under-developed	2. Under-developed
3. Developing	3. Developing
3.5	3. Developing
4. Advanced	4. Advanced
4.5	4. Advanced
5. Mature	5. Mature
6. Spawned	6. Spawned
7. Atretic	7. Atretic

Ovarian development of fish conditioned for reproduction at BLC was based on external morphology criteria used by BLC workers. Fish which were assessed by workers at BLC hatchery as being not ready for hormone-induced spawning were culled (returned to the pond)

and the remaining fish were sampled for ovarian development using the ultrasound imaging classification index. The number of fish returned to the pond and assessed as fish not ready for hormone-induced spawning was recorded for one sampling date at BLC hatchery, but this data is not available for the other sampling dates at the BLC hatchery (Table 7.5).

Table 7.5 Dates of ultrasound assessments of channel catfish ovarian development at the Louisiana State University Agricultural Aquaculture Research Station in Baton Rouge, Louisiana (LA) and at Baxter Lands Company hatchery in Arkansas City, Arkansas (ARK) were scheduled based on a preliminary assessment and the two dates selected for transport of fish from LA to ARK for artificial spawning. The sampling identification (ID), the sampling date, the sampling location, and the average time (average ± SD) of ultrasound assessments were listed.

ID	Date	Location	Number of fish	Time/Fish
1	04/25/2008	LA	49	114 s ± 47 s
2	04/28/2008	LA	638	27 s ± 44 s
3	04/29/2008	LA	222	18 s ± 16 s
4	05/03/2008	ARK	60	59 s ± 25 s
5	05/12/2008	LA	638	30 s ± 53 s
6	05/16/2008	ARK	79	21 s ± 17 s
7	05/17/2008	ARK*	131	31 s ± 24 s
8	05/18/2008	ARK	118	21 s ± 23 s

A total of 1,935 ultrasound image assessments of channel catfish ovarian development were completed at the LSU AC-ARS in Baton Rouge, Louisiana and at BLC hatchery in Arkansas during eight sampling dates in April and May (Table 7.5). The time (average ± SD) needed for these fish handling and imaging procedures was recorded for each image. The majority of these assessments (77%) were completed during 3 days (Sampling ID 2, 3 and 5) with an average time of < 30 s between images recorded of each fish (Table 7.5). The average time for ultrasound assessments of fish in Arkansas was 21 to 31 s (Table 7.5).

Hormone-Induced Spawning and Artificial Fertilization. The fish that were transported from Louisiana to Arkansas on April 29, 2008 for artificial spawning were divided into three groups

designated as Batch 1, Batch 2, and Batch 3. Batch 1 was comprised of fish assessed as Categories 4 and 5 from pond B4. Batch 2 was comprised of fish assessed as Category 5 from pond B6. Batch 3 was comprised of fish assessed as Category 4 from pond B6. The fish that were selected for transport from Louisiana to Arkansas on May 12, 2008 for artificial spawning were divided into two batches designated Batch 4 and Batch 5. Batch 4 was comprised of fish assessed as Category 5. Batch 5 was comprised of fish assessed as Categories 3 and 4. Batch 6 was comprised of fish conditioned for reproduction at ambient temperatures in a 0.4-ha pond (Pond 1) at BLC hatchery. These fish were assessed as Categories 3, 4 and 5 using the ultrasound imaging reproductive index.

The fish were held in concrete raceways during artificial spawning at BLC hatchery. They were injected with Carp Pituitary Extract (CPE) with a priming dose of 2 mg CPE/kg of fish and a resolving dose of 8 mg CPE/kg of fish 14 h later. Fish were checked for egg release at 2 h intervals, and eggs were collected by manual strip-spawning. Blue catfish males were sacrificed, and the testis were extracted, weighed and mascerated to create a sperm suspension using Hanks' balanced salt solution (Avery et al. 2005). Eggs were poured into food-grade baking pans at 300-400 mL eggs/pan for Batch 1, 2, 3, 4 and 5 and at 300 – 600 mL / pan for Batch 6. A 5-mL volume of blue catfish sperm was added to each pan and the gametes were activated with hatchery water. After egg cohesion, the egg mass was placed in flow-through raceways containing mesh baskets and paddlewheel aerators for circulating the water and for movement of the egg mass.

Fertilization Estimates and Egg Quality. Three portions of the egg masses were illuminated by placing a waterproof flashlight under the egg mass. The number of eggs undergoing embryonic development were counted for 10 eggs in each of the three illuminated portions of the egg masses (n = 30 eggs/egg mass). Embryo development was assessed by visually identifying the neural tube formation (approximately 30 h after activation of gametes of eggs incubated in water at 26 C) visible as a line when illuminated with a light source (Pawiroredjo 2004).

The number of eggs that progressed to neurulation was counted for all the batches sampled except Batch 1, which was assessed at 48 to 56 h during embryo mobility. Fertilization was estimated as the number of eggs undergoing embryo development divided by the total number of eggs counted for each egg mass multiplied by 100 to obtain a percentage. The egg quality was considered low if the fertilization estimate was < 50%, and high if the fertilization estimate was ≥ 50% (Bates and Tiersch 1998).

Statistical Analysis. The 'Proc Logistic' model procedure of the Statistical Analysis Software (SAS) system version 9.3 for Microsoft® Windows® (SAS Institute Inc., 2012, Cary, NC, USA) was used to test for differences in ovarian development of fish conditioned for reproduction at ambient temperatures in Louisiana and Arkansas. Ultrasound Categories 4 (Advanced) and 5 (Mature) were used in the statistical comparison of ovarian development for these two locations because of their relevance to commercial production of viable eggs.

The 'Proc Frequency' model procedure of SAS was used to test for differences in the proportions of low (< 50% fertilization estimate) and high (≥ 50% fertilization estimate) egg quality of fish

selected for artificial spawning in commercial hatchery production. First, the "Proc Frequency' procedure was used to test for differences in egg quality of fish assessed as Categories 4 and 5 in Louisiana and selected for artificial spawning in Arkansas.

Batches 1, 2 and 3 were used in this statistical analysis because these fish were evaluated using ultrasonography and transported during the same dates for hormone injection to ensure any transportation effect was the same; (ii) because these batches were comprised of fish assessed as Categories 4 and 5; and (iii) because these batches were kept in separate traceable groups from the time of ultrasonography in Louisiana to the time of fertilization assessment in the commercial hatchery troughs in Arkansas. The 'Proc Frequency' procedure was also used to test for differences in egg quality of fish conditioned in Louisiana and in Arkansas which were selected for artificial spawning. Differences for all statistical analysis were considered significant at $P < 0.05$.

Results

The thermal profile of the broodstock ponds and the ultrasound imaging data collected during the eight sampling periods in this study showed that ovarian development of channel catfish in Louisiana was more advanced compared to the ovarian development of broodfish conditioned for reproduction at ambient temperatures in Arkansas at the same time (Figure 7.1, Tables 7.6 and 7.7). Ambient temperatures of ponds at the LSUAC-ARS in Louisiana was constantly higher and reached 21 C (the threshold temperature used for degree-day calculations of heat accumulation for spawning) four weeks earlier (March 28) than at BLC hatchery in Arkansas (Figure 7.1). During the first ultrasound imaging assessment of fish in Louisiana (Sampling ID 1, Table 7.5), the degree-day profile measuring reproductive readiness for spawning was 84 dd.

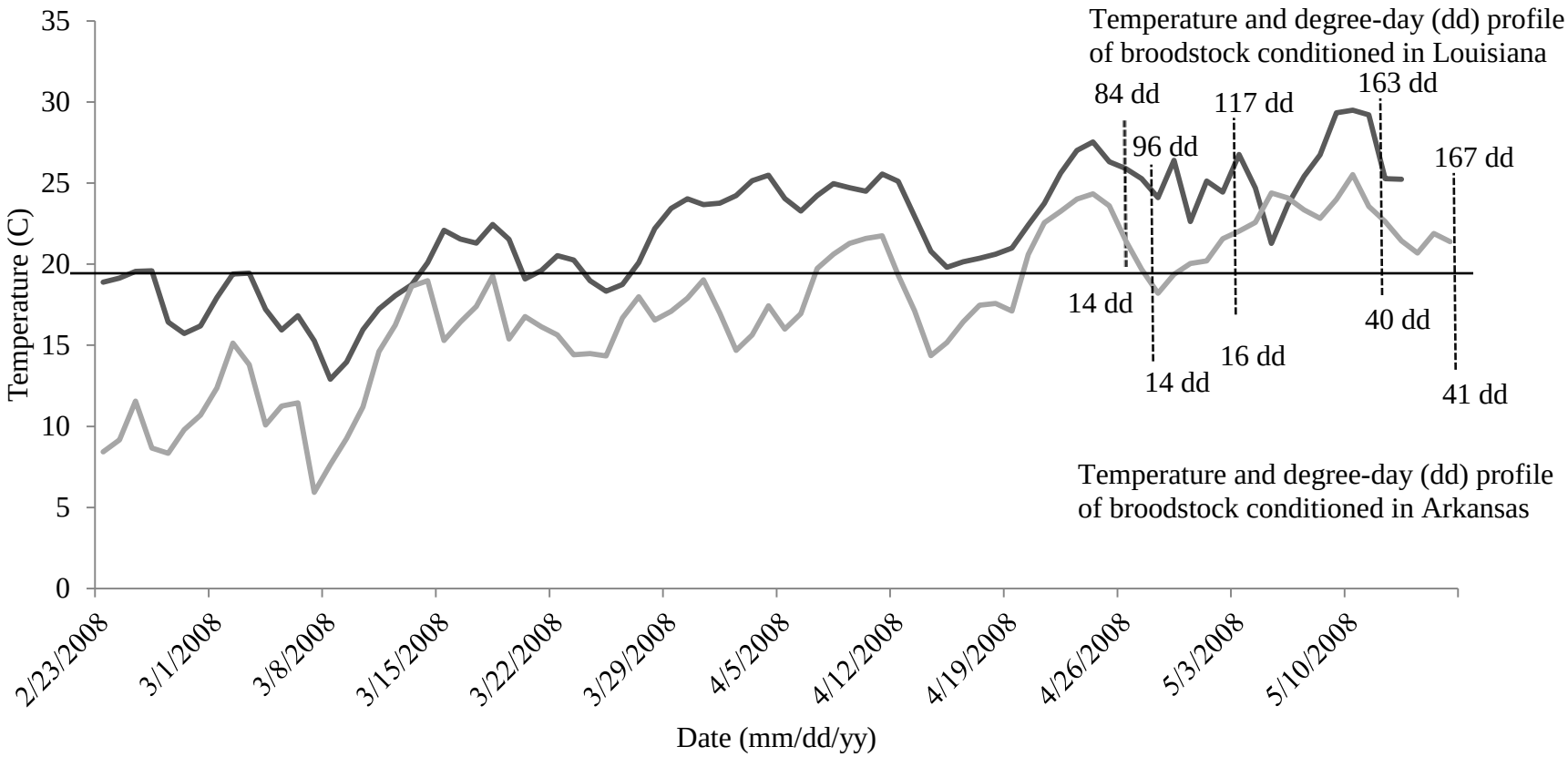


Figure 7.1 The temperature profile (C) for broodstock ponds (0.30-ha) at the Louisiana State University Agriculture Center Aquaculture Research Station in Louisiana (black line) and broodstock ponds (2.55-ha) from which fish originated at Baxter Lands Company hatchery in Arkansas (grey line). The degree-day measure of reproductive readiness for spawning were calculated for the ultrasound imaging sampling dates (dashed line) of ambient water temperatures in Louisiana (the number above the dashed line) and in Arkansas (the number below the dashed line).

This thermal profile indicated that fish in these ponds were beyond the standard degree-day guideline developed for the onset of spawning (10% spawning, 57 – 81 degree-days) (Pawiroredjo et al. 2008). Ovarian development of 41% (n = 20 fish) were assessed as Category 4 and 5 of the total number of fish sampled (n = 49) during the preliminary sampling date at the LSUAC-ARS in Louisiana (Sampling ID 1, Table 7.5 and 7.6). The degree-day profile of the ponds at BLC hatchery from which the broodstock conditioned at LSUAC-ARS originated was 14 degree days at the same time, indicating that the spawning period had not started (Figure 7.1). This preliminary assessment led to the decision to assess all the fish in ponds B4 and B6 at LSUAC-ARS to select fish to be transported to Arkansas on April 29, 2008 for artificial spawning.

Based on the degree-day calculations and the number of fish assessed as Categories 4 and 5 of the ultrasound imaging classification index, more fish were ready for artificial spawning at an earlier date at the LSUAC-ARS (n = 313 fish) than fish (n = 32 fish) at BLC hatchery in Arkansas during the first set of sampling dates (Figure 7.1, Table 7.6). Fish from ponds B4 and B6 selected for transport to Arkansas 2 weeks later had a thermal profile of 163 degree-days, which indicated that fish were near the conclusion of spawning (90% spawning, 150 – 172 degree-days). At the same time, the degree-day profile of broodstock pond at BLC hatchery from which the fish conditioned at the originated was 40 dd, which was before the standard degree-day guideline developed for the onset of spawning (Pawiroredjo et al. 2008).

Table 7.6 Ultrasound assessments during the first set of sampling dates of fish conditioned for reproduction at ambient temperatures at the LSUAC-ARS in Baton Rouge, Louisiana. Of the 860 fish collected by seine from ponds B4 and B6, 313 were assessed as Categories 4 (Advanced) and 5 (Mature). Of the 60 fish assessed at BLC hatchery in Arkansas City, Arkansas using ultrasound imaging after BLC workers had inspected and rejected fish considered not ready for artificial spawning, 32 fish were assessed as Categories 4 (Advanced) and 5 (Mature).

Date:	Apr 25	Apr 28	Apr 29	May 3
Location:	LSUAC-ARS			BLC
Pond:	B6	B6	B4	Pond 1
Ultrasound Imaging Categories	Number of Fish			
1. Undeveloped	6	40	6	-
2. Under-developed	6	65	13	-
3. Developing	17	327	88	28
4. Advanced	10	109	58	20
5. Mature	10	95	51	12
6. Spawned	0	0	5	-
7. Atretic	0	0	1	-

Table 7.7 Ovarian ultrasound assessments during the second set of sampling dates was of all the fish stocked into two ponds and conditioned for reproduction at ambient temperatures at the LSUAC-ARS in Baton Rouge, Louisiana. Ovarian development of fish conditioned at ambient temperatures in ponds at BLC hatchery in Arkansas City, Arkansas was assessed first by BLC workers based on external morphology. Fish judged as not ready for artificial spawning were returned to the pond. Subsequent ultrasound imaging assessments of ovarian development were of the fish not returned to the ponds.

Date:	May 12		May 16	May 17	May18
Location:	LSUAC-ARS			BLC	
Pond:	B6	B4	Pond 1	Pond 41*	Pond 3
Ultrasound Imaging Categories	Number of Fish				
1. Undeveloped	80	40	-	-	0
2. Under-developed	65	14	-	3	0
3. Developing	172	61	35	45	2
4. Advanced	54	38	20	44	32
5. Mature	39	49	24	39	82
6. Spawned	16	0	-	-	0
7. Atretic	10	0	-	-	2

*The number of fish (n = 131 fish) assessed using the ultrasound imaging classification index for pond 41 was the remaining fish after 981 fish were returned to the pond by BLC workers.

The statistical analysis comparing the ultrasound imaging ovarian development assessments of Categories 4 and 5 (Tables 7.6 and 7.7) conditioned for reproduction in Louisiana and in Arkansas showed that ovarian development was significantly dependent ($P < 0.05$) on the location used for reproductive conditioning.

Of the total number of fish (n = 1,112 fish) assessed for ovarian development by BLC workers, in Arkansas, 88% (981 fish) of were returned to Pond 41 during ovarian development assessments on May 17, 2008 (Table 7.7). The remaining 12% were assessed using the ultrasound imaging index (Table 7.7). Thus, of the total number of fish (n = 1,112 fish) in pond 41 assessed for ovarian development, 8 percent (n = 83 fish) was assessed as Categories 4 and 5 of the ultrasound imaging index (Table 7.7). In total, more than 3,050 ovarian development assessments were completed during this study, including the assessments made by external examination of fish by BLC workers.

Selection of Fish for Artificial Spawning. A total of 516 fish were selected for artificial spawning during commercial scale assessments of ovarian development (Table 7.8). Of these fish, 434 were conditioned for reproduction at the LSUAC-ARS. All the fish (Batch 1, 2, and 3) transported to Arkansas on April 29, 2008 for artificial spawning were assessed as Categories 4 and 5 (Table 7.5 – 7.8). The majority (81%) of the fish (Batch 4 and 5) selected for transport to Arkansas on May 12, 2008 was comprised of Categories 4 (n = 67 fish) and 5 (n = 83 fish). However, 36 fish assessed as Category 3 were included in Batch 5 (Table 7.8).

Table 7.8 Batch 1 to 5 was comprised of fish conditioned for reproduction at ambient temperatures at the LSUAC-ARS that were selected for transport to BLC hatchery for artificial spawning. Batch 6 was comprised of fish conditioned for reproduction at ambient temperatures at BLC hatchery that were selected for artificial spawning.

Batch ID	Ultrasound imaging assessment categories	Number of fish injected	Weight (kg)	
			Average	Standard deviation
1	4 & 5	95	2.4	0.5
2	5	97	2.4	0.4
3*	4	56	2.3	0.5
4	5	83	2.3	0.5
5	3 & 4	103	2.0	0.5
6**	3, 4 & 5	82	2.2	0.5

*53 fish of the 109 fish designated as Batch 3 were culled by consultant assessor, Roger Yent.
** Two additional fish were injected but these were not assessed for ovarian development using ultrasonography.

Fertilization Estimates and Egg Quality. The fertilization estimate of the egg masses were highly variable and ranged between 0 to 90% for eggs collected from fish assessed as Categories 4 and 5 for fish conditioned in Louisiana and for fish assessed as Categories 3, 4 and 5 and conditioned for reproduction in Arkansas (Figure 7.2, 7.3). The proportions of low quality (< 50% fertilization estimate) to high quality (≥ 50% fertilization estimate) eggs amongst Batch 1, 2 and 3 were not significantly different ($P = 0.18$).

The proportions of low and high quality eggs were pooled for Batch 1, 2 and 3 to represent ultrasound imaging Categories 4 and 5 because these fish underwent the same transportation and artificial spawning conditions. This pooled data was compare to the proportions of low and high quality eggs collected from ovarian assessments made of fish selected by external examination of fish morphology by BLC workers. These fish were characterized as ultrasound imaging Categories 3, 4 and 5 (Figure 7.3, Table 7.6 and 7.8).

The number of egg masses with higher quality eggs were significantly different ($P = 0.03$) for fish assessed as Categories 4 and 5 (Batch 1, 2 and 3) conditioned for reproduction in Louisiana than the fish conditioned in Arkansas that were assessed as Categories 3, 4 and 5 (Batch 6).

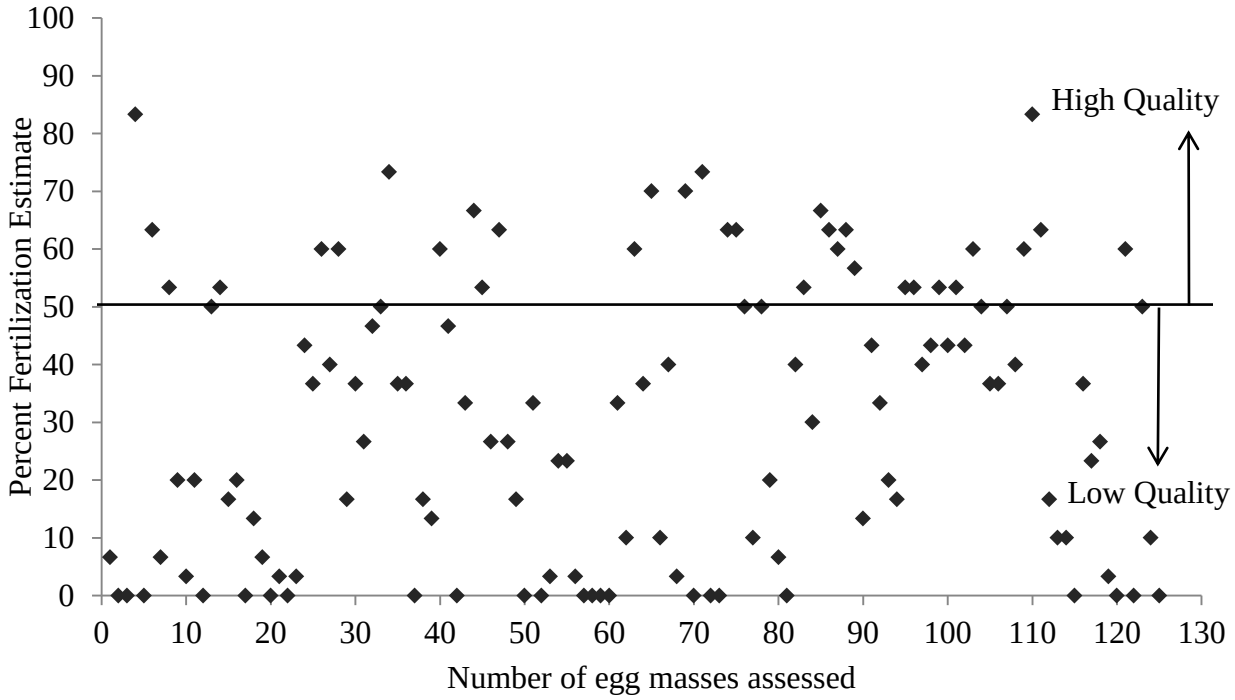


Figure 7.2 Fertilization estimate of egg masses (n = 125 egg masses; 300 – 400 mL eggs/egg mass) that were produced during artificial spawning at BLC hatchery in Arkansas of fish from Batch 1, 2 and 3. These fish were conditioned for reproduction at ambient temperatures at LSUAC-ARS in Louisiana and assessed as Categories 4 (Advanced) and 5 (Mature) of the ultrasound imaging classification index. Egg quality was ≥ 50% fertilization estimate for 39 egg masses (high quality eggs). Egg quality was < 50% fertilization estimate for 85 egg masses (low egg quality).

In total 208 egg masses of 300 to 400 mL each of channel catfish conditioned for reproduction in Louisiana, and 33 egg masses of 400 to 600 mL of fish conditioned for reproduction in Arkansas were artificially fertilized with blue catfish sperm at BLC hatchery for hybrid production.

Fish conditioned for reproduction in Louisiana and assessed as Categories 4 (Advanced) and 5 (Mature) produced a total of 164 egg masses (300 – 400 mL eggs/egg mass). Ninety masses were comprised of low (< 50%) quality eggs of 23 ± 14 % (average ± SD). Forty-eight masses were comprised of high (>50%) quality eggs of 60 ± 9 % (average ± SD), and 26 egg masses had 0% embryo development.

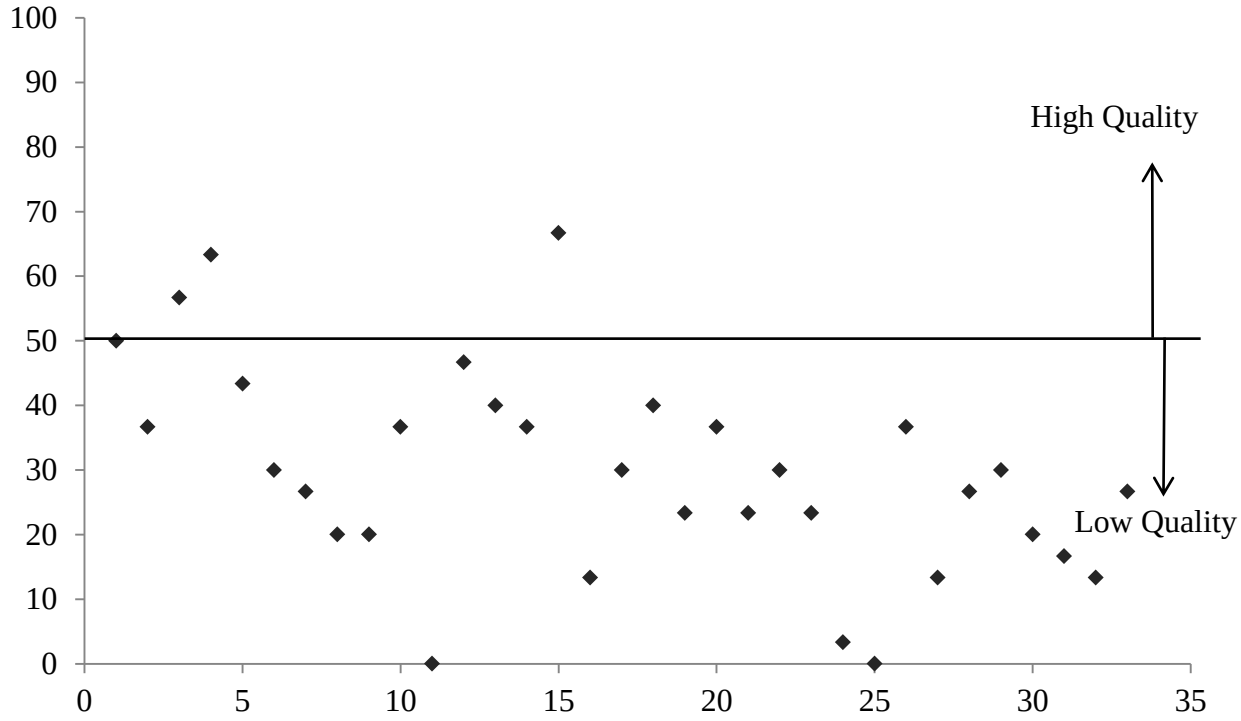


Figure 7.3 Fertilization estimate of egg masses (n = 33 egg masses; 300 – 600 mL eggs/egg mass) that were produced during artificial spawning at BLC hatchery in Arkansas of fish from Batch 6. These fish were conditioned for reproduction at ambient temperatures at BLC hatchery in Arkansas and assessed as were comprised of Categories 3 (Developing), 4 (Advanced) and 5 (Mature) of the ultrasound imaging classification index. Egg quality was ≥ 50% fertilization estimate for 4 egg masses (high quality eggs). Egg quality was < 50% fertilization estimate for 29 egg masses (low egg quality).

Fish conditioned for reproduction in Louisiana and assessed as Categories 3 (Developing) and 4 (Advanced) produced 44 egg masses. Thirty-three masses were of low (<50%) quality eggs of

26 ± 15 % (average ± SD). Seven masses were high (>50%) quality eggs of 55 ± 5 % (average ± SD), and 4 egg masses had 0 % embryo development.

In total, 33 egg masses (300 – 600 mL eggs/egg mass) were produced by fish conditioned in Arkansas and assessed as ultrasound imaging Categories 3 (Developing), 4 (Advanced) and 5 (Mature). Twenty-eight egg masses were low (<50%) quality eggs of 28 ± 11 % (average ± SD). Three masses were high (>50%) quality eggs of 62 ± 5 % (average ± SD), and 2 egg masses had 0 % embryo development (Figure 7.3).

Discussion

An ultrasound imaging categorical approach to assessment of ovarian development was used in commercial-scale evaluation of channel catfish for production of hybrid fry produced by crossing the female channel catfish and male blue catfish. The ultrasound imaging assessments were completed using the same standardized procedures in Louisiana and Arkansas. This included use of standardized fish handling and ultrasound imaging procedures, and use of the ultrasound imaging classification index. Based on these ultrasonographic assessments, an ovarian development profile was obtained of all the fish from two ponds (B4 and B6) at LSUAC-ARS to identify fish for artificial spawning at a commercial hatchery (Figure 7.4).

During sampling at the BLC hatchery in Arkansas, fish were selected for ultrasound imaging assessments after BLC workers had sorted through fish and culled those that did not meet their criteria for hormone-induced spawning. The number of fish (n = 985 fish, pond 41) returned to

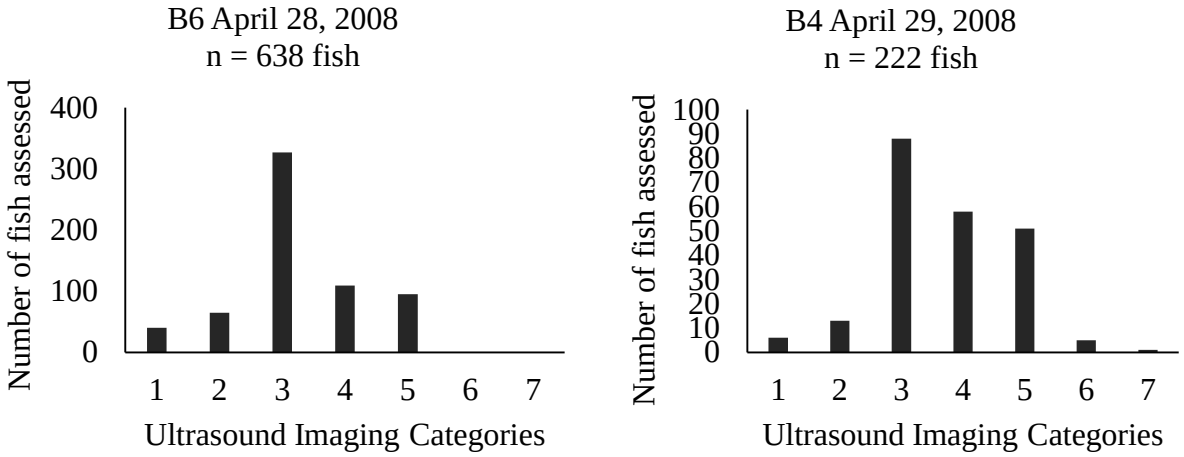


Figure 7.4 Ultrasound imaging assessment profile of ovarian development of channel catfish held in broodstock ponds at the LSUAC-ARS in Louisiana.

the pond by BLC workers indicated that a large number of fish was sorted and handled at BLC hatchery compared to fish captured by seine (n = 860 fish) on April 28 to 29, 2008 at the LSUAC-ARS to obtain fish for hormone-induced spawning.

The temperature data at BLC hatchery was obtained from ponds from which broodstock were captured six months earlier (November 2007) for transport for reproductive conditioning in ponds B4 and B6 at the LSUAC-ARS in Baton Rouge, Louisiana. Ovarian development as assessed using ultrasonography was more advanced in Louisiana. This was corroborated by the thermal profile. Fish in Louisiana were at 84 and 96 degree days. This indicated that 10 to 50% of the fish were expected to be ready for spawning based on the standard degree-day guidelines for the onset and median of spawning (Pawiroredjo et al. 2008). The temperature profile for the pond in Arkansas for all the sampling periods was 14 to 41 degree-days, which indicated that natural spawning activities had not started (Pawiroredjo et al. 2008).

From a commercial-scale perspective, the fundamental goal for the ultrasound imaging assessments were to select females for large-scale production of eggs for use in hybridization with male blue catfish for production of F_1 fry as seed stock. This study was designed to condition fish in Louisiana for this purpose. The first hypothesis was that these fish would be ready to spawn at an earlier date than fish conditioned for reproduction at Arkansas because of higher temperatures at this latitude. This provided an opportunity for thorough assessment of all the fish that were transported from BLC hatchery to the LSUAC-ARS to identify fish with advanced ovarian development for hormone-induced spawning at Arkansas.

The utility of the ultrasound imaging reproductive index for commercial hatchery production of hybrids was in identifying the fish with ovarian morphologies associated to the production of viable and unviable eggs. The hypothesis in relation to the ultrasound imaging categorical assessments were that fish assessed as Categories 1 (Undeveloped), 2 (Underdeveloped), 3 (Developing), 6 (Spawned) and 7 (Atretic) would not produce viable eggs. Viable egg collection was associated to Category 4 (Advanced) and 5 (Mature). Category 4 and 5, therefore, were the targeted ovarian reproductive assessments for selection of fish for transport to Arkansas for hormone-induced spawning.

Due to the logistics and commercial-scale aspect of this study, data for individual fish were not recorded, and groupings of ultrasound imaging assessments were used to identify fish selected for artificial spawning at BLC hatchery. Batch 1 was comprised of fish with ultrasound imaging Categories 4 and 5 and placed in one compartment of the hauler used to transport the fish from Louisiana to Arkansas. Batch 5 was comprised of fish with ultrasound imaging assessments 3

and 4 which were placed in the same trough system and not individually traced for fertilization assessment. Although these fish were not individually traceable, they comprised fish which would not have been available to the hatchery in Arkansas at that period of time and they provided commercial-scale egg production profile of the ultrasound imaging ovarian development assessments.

There were no statistical differences in the quality of eggs obtained from fish in Batches 1, 2 and 3 which were assessed as Categories 4 (Advanced) and 5 (Mature). Although the fertilization estimates were highly variable for all the fish which were selected for artificial spawning, The batches of fish conditioned in Louisiana contributed significantly more masses of high quality eggs from fish assessed as Categories 4 (Advanced) and 5 (Mature) of the ultrasound imaging classification index than fish in Batch 6 that were conditioned for reproduction in Arkansas and assessed as Categories 3 (Developing), 4 (Advanced) and 5 (Mature).

Summary and Conclusions

Based on the degree-day profiles of broodstock ponds and on the ultrasound imaging assessments of ovarian development in Louisiana and in Arkansas, fish conditioned for reproduction in Louisiana were ready for artificial spawning at an earlier date compared to fish held in ambient temperature ponds in Arkansas. Ambient pond water temperatures reached 21 C one month earlier in Louisiana than in Arkansas, demonstrating the potential for reproductive conditioning of fish at more southern latitudes such as in Baton Rouge, Louisiana. Ovarian development assessed in Louisiana was based on the ultrasound imaging classification index developed for channel catfish at LSUAC-ARS. In order to identify fish for artificial spawning in the BLC ponds in Arkansas, a combination of external assessments by BLC workers and

ultrasonography assessments were made to identify fish for artificial spawning from large numbers of fish. During one assessment period, >1,000 fish were assessed in this manner, demonstrating that fish were not ready for hormone induced spawning in Arkansas at a time when ovarian development of fish conditioned in Louisiana was more advanced. While the fertilization estimates of all the fish selected for artificial spawning was highly variable, ultrasound imaging assessments of Category 4 and 5 yielded the highest number of high quality egg masses. The number of fish producing viable eggs for hybrid production at an earlier date than was possible in Arkansas was from fish conditioned in Louisiana and assessed as Categories 4 (Advanced) and 5 (Mature) of the ultrasound imaging classification index.

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Chapter 8
Summary and Conclusions

Previous researchers at the Louisiana State University Agricultural Center-Aquaculture Research Station (LSUAC-ARS) in collaboration with the Louisiana State University School of Veterinary Medicine laid the groundwork for studies of ultrasonography in channel catfish reproduction. Their work established initial fish handling and ultrasound imaging procedures during 2004 to 2005 for viewing the ovary of channel catfish *Ictalurus punctatus* (Guitreau et al. 2012). These handling and imaging techniques were used in this dissertation to develop a standardized and systematic approach that incorporated biological and technological components to interpret ovarian ultrasound images of the channel catfish. This led to the development of a standardized and well-defined ultrasound imaging classification index. As groundwork to begin this project, this dissertation first reviewed the scientific literature to evaluate the utility of ultrasonography in fish reproduction, and identified the needs for adaptation and standardization of this technology in fish reproduction.

1. Groundwork. The Literature Review (Chapter 3). The review of the use of ultrasonography in fish reproduction was intended to provide a global accounting of the procedures, equipment, methods of reporting, and research findings to serve as a decision-making tool for users of this technology. The review revealed that ultrasound imaging was used for sex identification and for the development of reproductive indices for freshwater species, and marine and anadromous fishes (Novelo and Tiersch 2012). These studies were conducted on male and female fishes. But ultrasonography was used on a larger number of females in species such as the Murray cod *Maccullochella peelii* (> 300 females in one study), red hind *Ephinephelus guttatus* (> 600 females in one study) and the anadromous form of the rainbow

trout *Oncorhynchus mykiss* (> 1,000 females in one study). Ultrasonography was also used in fisheries management studies on endangered fish such as the Neosho madtom *Noturus placidus* and pallid sturgeon *Scaphirhynchus platorynchus*, and in aquaculture studies of fish such as coho salmon *O. kisutch* and Atlantic halibut *Hippoglossus hippoglossus*.

Two major needs in the use of ultrasonography in fish reproduction were exposed by the review. The first was the need for standardization of fish handling and imaging techniques starting with particular species and fish of similar sizes. The second was the need for improved reporting of the ultrasound control settings used to obtain data in fish reproduction studies. These two needs were linked to the ability or inability to replicate findings in different studies, and to the underlying need to work towards mechanisms for standardization for maximizing the efficiency and utility of ultrasonography in aquaculture and fisheries.

A list of recommendations was provided to improve reporting and standardization of procedures that would enable comparison and replication of studies by novice or experienced users of this technology, especially those users coming to fish work from other fields such as animal science. The reporting of control settings used for obtaining ultrasound images was one basic recommendation because this was lacking in 89% of the studies reviewed, and in the remaining studies the settings were given for only one control such as 'Gain' and 'Power'. Another basic recommendation was to standardize and to improve reporting of ultrasound imaging and fish handling procedures. This included: (1) maintaining the fish in its aqueous environment to reduce stress and injury to the fish, and to enable the use of water as a conductive medium for the ultrasound waves (a significant improvement to exposing the fish to air); (2) the use of a

waterproof probe and a portable ultrasound unit; (3) the use of basic grey-scale imaging features (Real-Time B-Mode controls), and (4) the standardized use of defined image capture, processed image orientation and labeling (digital photographs or schematic diagrams) of the scanning region, and ultrasound image capture procedures.

2. Adaptation and Development of Ultrasound Technology for Use in Channel Catfish Reproduction. Standardized and Systematic Interpretation (Chapter 4). The systematic approach developed for interpretation of ultrasound images of the channel catfish ovary encompassed technical aspects of ultrasonography in combination with biological insights obtained during circannual observation and monitoring of the ovaries. One main component of this systematic approach to interpretation was recognizing the relative importance of the ultrasound control settings that govern the viewing field. This was evidenced by images of the same ovary produced by different suites of settings. One group of settings could produce a large-scale view of the ovary but did not provide resolution and clarity for observation of the oocytes. Another group of settings could provide a more in-depth view of the ovary and oocytes and the means with which to better observe morphological changes representative of biological processes in the ovary.

At the same time, ultrasound imaging artifacts caused by the airbladder and bone were identified and illustrated to assist in their recognition and avoidance. This ensured the display and recording of unobstructed images in which the shape, size and the echogenic appearance of the ovary and oocytes could be interpreted for their biological relevance to ovarian development. Clear ultrasound images were observed and associated with the processes of oocyte hydration

and maturation (ovulation of viable eggs), oocyte retention (no eggs released), and atresia (decomposition of non-viable oocytes). Thus, the biological insight gained from systematic interpretation of the images was based on: (1) technical mechanisms of ultrasonography; (2) understanding of the complex biological processes occurring in the ovary, and (3) the biological outcomes of artificial spawning.

The method of image illustration in reporting the interpretation of images was included as a mechanism to facilitate standardization in fish reproduction studies. This would not only ensure familiarity with the layout of the ultrasound images, but it would also assist in interpretation of the images portrayed and in increasing familiarity with the use of this technology in fish reproduction. This would also provide the benefit of enabling direct comparisons among published studies.

The Classification Index (Chapters 5 and 6). The core of this dissertation was the development of a standardized ultrasound imaging classification index as a decision-making tool for assessing channel catfish ovarian development. Chapter 5 presented the initial development, use and evaluation of the ultrasound imaging classification index. The preliminary development of the ultrasonography classification index was evaluated based on the agreement of the expected and observed outcome of seven categorical assessments of ovarian development during ten spawning trials from 2008 to 2010 (Chapter 5). Categories 1 (Undeveloped), 2 (Underdeveloped), 3 (Developing), 6 (Spawned) and 7 (Atretic) were expected to produce non-viable eggs, if any, during artificial spawning. Categories 4 (Advanced) and 5 (Mature) were expected to produce viable eggs during artificial spawning.

The analysis of the expected and observed outcomes showed that the outcome (production of viable or unviable eggs) was significantly associated to the ultrasound imaging classification assessments. An error matrix was tabulated to define the specific relationship of the expected and observed outcomes of each of the seven categories comprising the index. This analysis showed that the ultrasound imaging index was effectively used for identifying fish which would not produce viable eggs, and for maximizing the number of fish which would produce viable eggs. This was recognized as providing potential benefit to catfish hatcheries in selection of female broodstock suitable for hormone injection.

Chapter 5, along with the studies done by Guitreau et al. (2012) and the studies in Chapters 3 and 4 of this dissertation, provided the foundation for the establishment of a standardized and well-defined ultrasound classification index presented as a Reference Guide in Chapter 6. This guide included histological and ultrasound images of the same ovary selected to represent the seven categories developed. The classification index was the outcome of a developmental pathway towards standardization in discovering the biological significance of the ovarian images collected. It involved designing a template which was representative of the channel catfish ovarian cycle throughout the year, and more than 6,000 channel catfish were studied for its development.

Commercial Use (Chapter 7). Close collaboration with a commercial hatchery in Arkansas explored the utility of ultrasonographic assessment of ovaries in channel catfish at a commercial scale. This collaboration provided the opportunity to assess large numbers of channel catfish

(> 600 catfish females per day) at an average of about 30 sec per fish to identify females suitable for hormone injection and artificial spawning. This study showed that it was possible to start thermal reproductive conditioning in more southern latitudes such as in Baton Rouge, LA at an earlier date (in this study one month in advance) than at northern latitudes represented by Baxter Lands Company hatchery in Arkansas City, Arkansas.

The temperature profile defining the channel catfish reproductive readiness in ponds at the LSUAC-ARS was 96 degree-days, which represented the onset and median of spawning (Pawiroredjo et al. 2008). The temperature profile of ponds at the commercial hatchery in Arkansas at the same time was 14 degree days, which indicated that spawning activity had not begun (Pawiroredjo et al. 2008).

This ovarian development reproductive profile of channel catfish conditioned in Louisiana and in Arkansas was captured by use of the ultrasound imaging classification index. The ultrasound imaging profile of catfish at the LSUAC-ARS was generated. Based on this, females were selected for transport to Arkansas for artificial spawning. Ultrasound imaging assessment Categories 4 (Advanced) and 5 (Mature) yielded the highest number of high quality egg masses. This was the first commercial-scale study to use ultrasound imaging as an evaluation tool to decide whether to select females for hormone-induced spawning.

3. Pathways to Application of Ultrasound Technology. In the hypothetical scenario that envisions the utility of ultrasonography in channel catfish reproduction, the ability to assess ovarian development in a decisively discriminatory manner using the seven classifications of the

ultrasonography index provides a more efficient use of available resources and associated economic investments than using the two classifications (suitable or not suitable) of the manual examination of external morphology (Figure 8.1). The use of ultrasound would increase control and efficiency of broodstock management, hormone injection, and artificial spawning to attain the desired fry production goal in a shorter work period and in more effective selection of fish throughout the work period (Figure 8.1).

Females (Categories 1 Undeveloped; 2, Under-Developed; and 3, Developing) that were not yet ready for artificial spawning could be moved to a pond for spawning in several weeks instead of injecting these fish and wasting their spawning value for that spawning season (Figure 8.1). Females that would not produce viable eggs (Category 6, Spawned; Category 7, Atretic) could be placed in a pond for the following year's spawning season (Figure 8.1). These ultrasound assessments of ovarian development discriminating between vitellogenic, developing ovaries (Categories 1, 2 and 3) and spawned or atretic ovaries (Categories 6 and 7) (not possible using manual external examination alone) would thus enhance the monitoring of ovarian development and increase control of broodstock management and fry production (Figure 8.1).

The ultrasound assessments would provide increased efficiency in hormone injection and artificial spawning primarily by identifying fish that would not produce viable eggs,

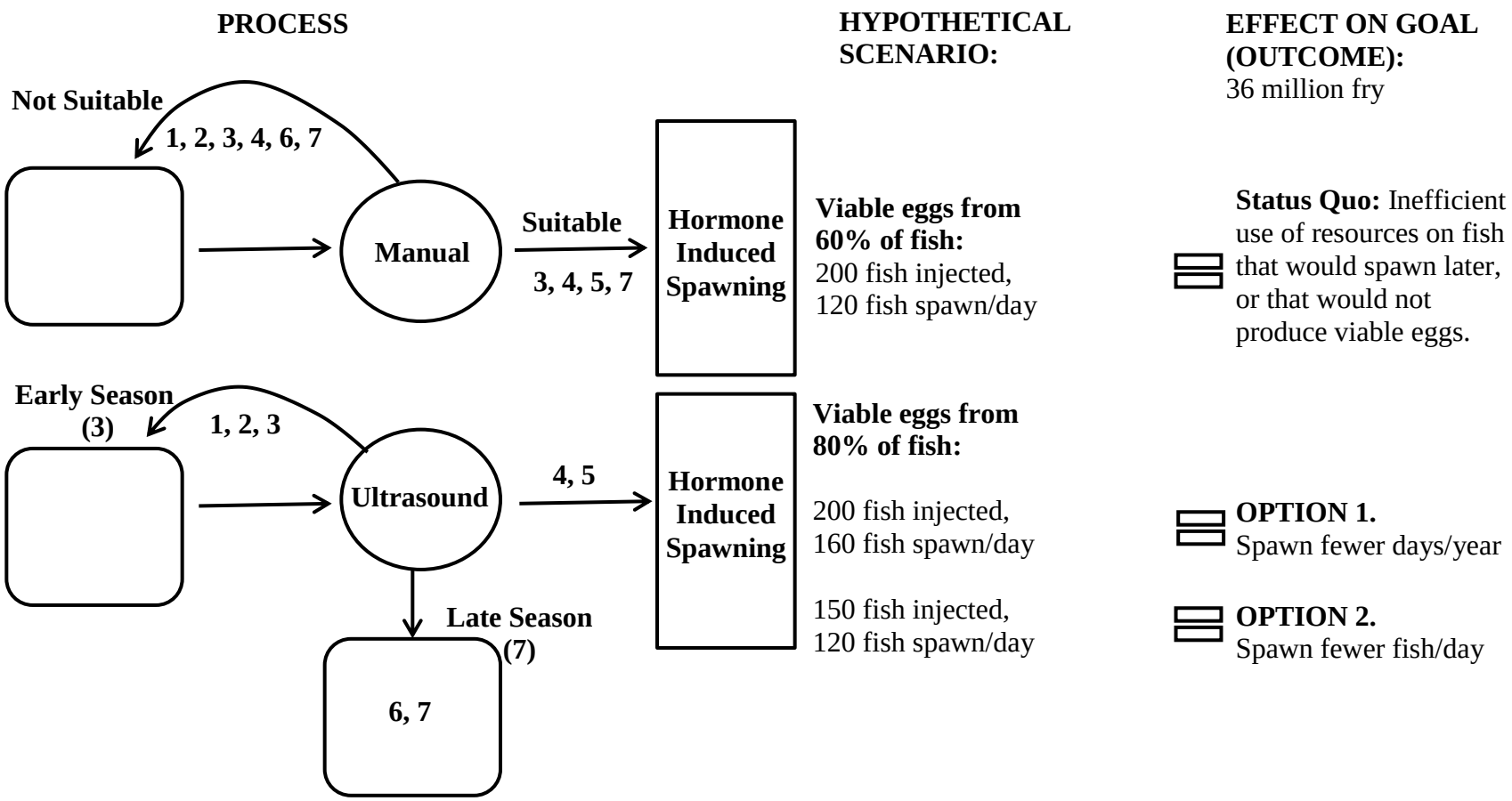


Figure 8.1 In this hypothetical scenario, using the external examination (manual) process, 60% of fish spawn; 10,000 eggs/fish x 120 fish = 1.2 million eggs/day at 50% survival to fry = 600,000 fry/day x 60 days = 36 Million Fry (Outcome). This same outcome could be achieved by use of ultrasound technology with increased control and efficiency in viable egg collection and use of broodstock resources. The gain would be in the removal of fish that would not produce viable eggs (Categories 3 and 7), and selection of fish that would produce viable eggs (Categories 4 and 5). This would provide different management options to achieve the fry production goal. The first would be to spawn fewer days per year, potentially eliminating days in the first and last weeks of the work season. The second would be to spawn fewer fish per day and obtain the same target within the 60 day work period.

(Categories 1, 2, 3, 6 and 7) and maximizing the number of fish that would produce viable eggs (Categories 4 and 5) throughout the spawning season (Figure 8.1). In the hypothetical scenario presented, selecting 200 fish for hormone injection and artificial spawning (resulting in viable eggs from 120 fish, i.e. 60% spawning efficiency) would include fish in Categories 3 and 7 by use of the external examination (manual process) that would otherwise have been excluded by use of direct examination of ovarian morphology based on ultrasound imagery (Figure 8.1).

The use of the ultrasound imaging classification index would provide beneficial management and efficiency options (Figure 8.1). Option 1 would be to inject the same number of fish (i.e. 200 fish), but with a higher efficiency rate, and thus obtain the fry production goal in fewer work days of the spawning season (Figure 8.1). This would be advantageous in the first weeks of the spawning season when ovarian development Category 3 (Developing) would likely be in high occurrence, identified, and moved to a pond for spawning later that season (Figure 8.1). Identification of fish with ovarian development Category 7 (Atretic) would be advantageous throughout the spawning season, but especially so in the last few weeks of the spawning season when more fish with Category 7 ovarian development are present. These fish would be identified and thus not selected for injection even if the fish's external appearance is of an enlarged ovary which may appear suitable for spawning but was not (Figure 8.1). This would concentrate the time and resources on the fish which would produce viable eggs and avoid wasting fish with developing ovaries not yet ready for spawning.

Option 2 would be to inject fewer fish (e.g. 150 fish instead of 200) and still contribute to the target fry production goal of the spawning season. These options would be based on the gains

provided by use of ultrasound to assess ovarian development changes occurring during the spawning season. This improved broodstock management and spawning efficiency would in turn produce savings in the time, labor, equipment, supplies, and blue catfish sacrificed for used in spawning efforts. Improved selection of females would mean more efficient use of valuable blue catfish *Ictalurus furcatus* sperm, and reducing the wastage of sperm on non-viable eggs. Removing immature fish or atretic fish by use of ultrasound would increase the efficient use of hatchery space as well. This would eliminate the non-viable eggs that, if put in the hatching troughs, would unnecessarily occupy space and degrade water quality.

4. Future Directions. Improved reproduction efficiency in catfish aquaculture could be addressed further by use of a technology platform that would include the use of (i) ultrasound imaging technology (Novelo et al. 2011; Novelo and Tiersch 2013) (ii) geothermal reproductive conditioning technology (Hall et al. 2002; Lang et al. 2003; Lang and Tiersch 2007), (ii) standardized degree-day guidelines to compare reproductive readiness of different ponds in different thermal regimes (Pawiroredjo et al. 2008), and commercial-scale cryopreservation of blue catfish sperm (Hu et al. 2011). The improved efficiency in broodstock management and reproduction provided by this platform would address genetic improvement not only in the present short-term solution provided by the female channel catfish and male blue catfish hybrid, but also by contributing to the production of genetically improved channel catfish.

In conclusion, this work has provided information necessary for use of ultrasonography in channel catfish reproduction for research and commercial hatchery use. This was based on the standardization of mechanisms for the adaptation and use of this imaging technology in fish

reproduction. These mechanisms included the use of consistent fish handling and imaging techniques, the development of standards for the reporting of control settings, illustration of figures, systematic interpretation of ultrasound images, and the consequent development of an illustrated guide to ovarian development. This standardization approach was intended to provide mechanisms for working towards maximizing the potential contribution of ultrasonography in fisheries and aquaculture reproduction.

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Appendix A
Standard Operating Procedures

These standard operating procedures (SOPs) originated from previous research conducted at the Louisiana State University Agricultural Center Aquaculture Research Station and were adapted from Hu 2012, Pawiroredjo 2004, Lang 2001, and Bates 1997.

Hu, E. 2012. High-throughput sperm cryopreservation of aquatic species. Dissertation, Louisiana State University.

Pawiroredjo, P. A. 2004. Temperature effects on spawning and fingerling production of channel catfish *Ictalurus punctatus*. Masters thesis, Louisiana State University.

Lang, R. P. 2001. Induction of early out-of-season spawning in channel catfish *Ictalurus punctatus*. Masters thesis, Louisiana State University

Bates, M.C. 1997. Production of transgenic channel catfish. Dissertation, Louisiana State University.

SOP-1. Hanks' balanced salt solution (HBSS)

Hanks' balanced salt solution is used as a collection medium for eggs during artificial spawning. The HBSS should be prepared with an osmolality of 290 to 300 mOsmol/Kg to prevent activation of the eggs.

Procedure:

1. Combine the ingredients (Table A-1) with distilled water; bring the total volume to ~ 3.9 L.
2. Stir until all solutes are dissolved.
3. Verify the osmolality of mixture using osmometer; adjust to 300 mOsmol/kg by adding water.
4. Distribute the solution into 1-L bottles, and label with the date and name of person preparing the solution.
5. Store bottles in refrigerator.

Table A.1 Ingredients for Hanks' balanced salt solution.

Ingredient	grams/Liter	Molarity
NaCl	8.00	0.1400
KCl	0.40	0.0050
CaCl2•2H2O	0.16	0.0010
MgSO4•7H2O	0.20	0.0010
Na2HPO4	0.06	0.0004
KH2PO4	0.06	0.0004
NaHCO3	0.35	0.0040
C6H12O6 (glucose)	1.00	0.0060

Tiersch, T. R., Goudie, C. A. & Carmichael, G. J. 1994. Cryopreservation of channel catfish sperm: storage in cryoprotectants, fertilization trials, and growth of channel catfish produced with cryopreserved sperm. *Transactions of the American Fisheries Society*, 123: 580-586.

SOP-2. Hydration and injection of leuteinizing hormone-releasing hormone analog (LHRHa).

The LHRHa is in freeze-dried powder form and is used to induce ovulation in female channel catfish.

1) Dissolve the LHRHa powder using distilled deionized bacteriostatic water to yield a final concentration of 1 mg / mL.

2) Administer the hormone by intraperitoneal injection behind the pectoral fin at a dose of 100 μg LHRHa / Kg body weight. This was done by use of one injection in Chapter 4 of this dissertation, and by use of two injections (a priming dose of 20 μg, and a resolving dose of 80 μg) in Chapter 5 of this dissertation.

SOP-3. Hydration and injection of Carp Pituitary Extract (CPE).
The CPE is in dried powdered form and is used to induce ovulation in female channel catfish.

1) Dissolve the CPE dried powder using distilled deionized bacteriostatic water to yield a final concentration of 10 mg / mL.

2) Administer the hormone by intraperitoneal injection behind the pectoral fin with a priming dose of 2 mg CPE/Kg body weight and a resolving dose of 8 mg CPE/Kg body weight. This procedure was used in one spawning trial in Chapter 5 of this dissertation.

SOP-4. Hand-stripping of eggs from female channel catfish
1) Anesthetize the fish with 150 to 200 ppm of tricaine methanesulfonate (Western Chemical Inc., WA, USA).

2) Remove fish from anesthetic. Minimize release of eggs by holding the fish so the genital opening faces upward, and gently pat the fish dry using paper towels to avoid activation of the eggs with water.

3) Rinse hands with HBSS. Rotate the fish so that the genital opening faces downward. Hold the caudal peduncle of the fish with the weaker arm and support the body of the fish with the forearm of the stronger arm. Apply gentle pressure upwards and towards the oviduct. At no time should excessive pressure be applied as this can damage the ovaries and obstruct the oviduct.

4) Collect eggs into a greased bowl containing 100 mL of HBSS. The bowls coated with a thin layer of high vacuum grease (Dow Corning® high vacuume grease, SPI Supplies, West Chester, PA, USA) to prevent the eggs from sticking to the bowl. The HBSS is used to prevent activation of the eggs by contamination with water. Eggs should remain covered with HBSS at all times. If blood appears when stripping eggs, remove the liquid and replace with fresh HBSS. Remove any blood clots and ovarian tissue from the bowl.

5) When stripping is complete, revive the fish in fresh water and note the volume, color, size, and shape of the eggs.

SOP-5. Collection and incubation of channel catfish egg masses from pond spawning containers for egg masses data in Chapter 5 of this dissertation.

1) Turn off and disconnect the aerator from the power box.

2) Carefully locate the spawning container and tap it with your foot to encourage the male to leave the container. Males guarding the nest and can get trapped in the container and can damage the egg mass.

3) Lift container such that the opening faces upward. Slowly drain the water from the container while pivoting it over a knee for support. Periodically look inside the container to locate the mass.

4) Gently scrape the mass with the palm of your hand or with a food-grade plastic spatula from the wall of the container. Place the mass into a 4-L cooler with pond water and close the lid. Use a portable Bubble-box aerator (Bubble Box ™ B-11, Marine Metal Products, Clearwater, Florida) to aerate the water in the cooler.

5) Transport the egg mass to the hatchery within 30 min of collection and acclimate them for 15 min to hatchery water temperatures.

6) Weigh the egg mass and disinfect it by dipping in a 10% Iodine solution (Argentyne, Argent Chemical Laboratories, Redmond, Washington) for 10 min.

7) Break the mass into ~ 250 g portions and incubate them in aerated recirculating system containing water at 27 C. Plastic baskets can be used to suspend the mass adjacent to the airstone.

8) Count the number of eggs in three weighed samples of the egg mass.

9) Percent fertilization (SOP 6) is estimated by examining three random portions of the mass for fertilized and unfertilized eggs.

SOP-6. Estimation of fertilization of eggs (percent neurulation).

1) Pour a monolayer of eggs into the bottom of 100-mL tri-corner plastic beakers (Thermo Fisher Scientific Inc., Suwanne, GA, USA).

2) Add 0.5-mL of sperm to the eggs and activate the gametes with 10-mL of hatchery water.

3) Wait 5 to 10 min to add 10-mL more water and allow the eggs to harden and form a mass.

4) Incubate the monolayer egg mass into a plastic pipe with holes drilled in the side to allow water circulation. The holes on the sides and bottom of the pipe should be covered with glued mesh screen. The hole on the top of the pipe should be covered with a mesh screen held by rubber bands. These monolayer incubation units should be placed in a basket that is suspended off the bottom of the hatching trough.

5) At 30 h following activation examine the mass for the presence of unfertilized eggs. They will appear white or clear and are often swollen. Count the number of unfertilized eggs.

6) Count the number of neurulated eggs in the same sample. The neurulated eggs can be identified by the formation of the neural tube (Figure A.1) The neural tube becomes visible as a line when illuminating the egg monolayer with a hand held light from the bottom of the plastic container, and it is clearly visible under microscopic examination (Figure A.1). Estimate the percentage of fertilization based on the number of unfertilized eggs and neurulated eggs.

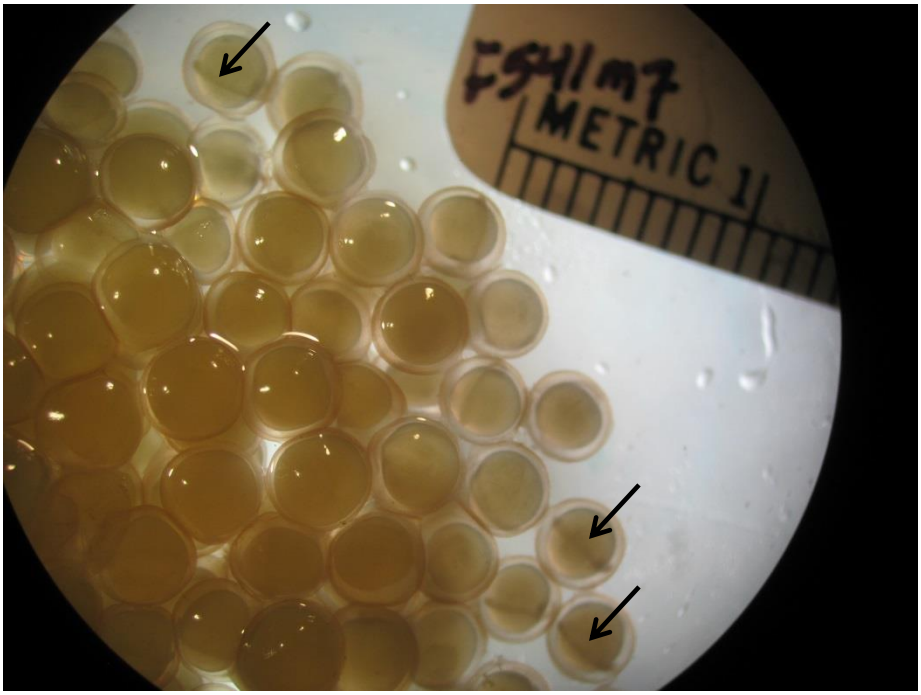


Figure A.1 Digital image of neurulation was obtained using a stereoscope (Nikon SMZ-U, Tokyo, Japan). A mm-increment ruler was positioned in the upper right corner. Arrows indicate examples of the neurula stage.

Appendix B
Data and Statistics Used in Research Chapters

The data and statistics used in Chapters 4, 5, and 7 were formatted for analysis with the Statistical Analysis Software (SAS) system version 9.3 for Microsoft® Windows® (SAS Institute Inc., 2012, Cary, NC, USA).

Data and statistics used for Chapter 4.

Statistical Analysis 4.1 to 4.4 tested the fixed effects of 'Temperature,' 'PondSex,' and 'TankPair' and their interaction on spawning latency and spawning rate. Statistical Analysis 4.5 and 4.6 tested an additional fixed effect, 'SpermUsed,' on fertilization. Statistical Analysis 4.7 tested the effect of the different males (MID) used on fertilization. The temperature of ponds was either heated (H) using geothermal water or ambient (A) water temperature (the 'Temperature' fixed effect). Ponds were either stocked with female and male (Mixed) fish or only with female (F) fish (the 'PondSex' fixed effect). Fish were placed in spawning tanks in either male-female (M-F) or female-female (F-F) pairs (the 'TankPair' fixed effect). Four concentrations of sperm were used during the spawning trials tested (the 'SpermUsed' fixed effect). Three males sets of males were used for each spawning trial tested (the 'MID' fixed effect).

Statistical Analysis 4.1. The spawning latency and fixed effects data analysis with the generalized linear mixed model (GLIMMIX) SAS procedure without the 'PondSex' variable in the model statement was as follows:

```
dm 'log;clear;output;clear';
title1 "test for differences in latency, without pondsex";
options nodate nocenter pageno=1 ls=78 ps=55;
ODS listing;
Data Latency;
Input TP$ FID$ L Temp$ TankPair$ PondSex$;
Datalines;
I       F16     53      H       M-F     Mixed
I       F19     43      H       M-F     Mixed
I       F21     55      H       M-F     Mixed
I       F24     64      H       F-F     Mixed
II      F48     45      H       F-F     F
II      F43     49      H       F-F     F
II      F45     78      H       F-F     F
III     F64     46      H       F-F     Mixed
III     F70     54      H       M-F     Mixed
IV      F76     50      H       F-F     F
IV      F79     42      H       M-F     F
IV      F84     49      H       F-F     F
V       F104    51      A       F-F     F
V       F110    45      A       M-F     F
```

```
V       F100    44      A       F-F     F
VI      F112    32      A       F-F     Mixed
VI      F113    33      A       F-F     Mixed
VI      F114    31      A       F-F     Mixed
VI      F116    32      A       M-F     Mixed
VI      F117    34      A       M-F     Mixed
;
PROC GLIMMIX DATA = Latency;/*without PondSex*/
CLASS PondSex FID TankPair Temp TP;
MODEL L = TEMP|TankPair / DIST = poisson solution LINK = LOG;
Random _Residual_ /Subject = TP;
Run;
quit;
```

Statistical Analysis 4.2. The spawning latency and fixed effects data analysis with the generalized linear mixed model (GLIMMIX) SAS procedure without the 'Temperature' variable in the model statement was as follows:

```
dm 'log;clear;output;clear';
title1 "test for differences in latency, without temp";
options nodate nocenter pageno=1 ls=78 ps=55;
ODS listing;
Data Latency;
Input TP$ FID$ L Temp$ TankPair$ PondSex$;
Datalines;
I       F16     53      H       M-F     Mixed
I       F19     43      H       M-F     Mixed
I       F21     55      H       M-F     Mixed
I       F24     64      H       F-F     Mixed
II      F48     45      H       F-F     F
II      F43     49      H       F-F     F
II      F45     78      H       F-F     F
III     F64     46      H       F-F     Mixed
III     F70     54      H       M-F     Mixed
IV      F76     50      H       F-F     F
IV      F79     42      H       M-F     F
IV      F84     49      H       F-F     F
V       F104    51      A       F-F     F
V       F110    45      A       M-F     F
V       F100    44      A       F-F     F
VI      F112    32      A       F-F     Mixed
VI      F113    33      A       F-F     Mixed
VI      F114    31      A       F-F     Mixed
VI      F116    32      A       M-F     Mixed
VI      F117    34      A       M-F     Mixed
;
```

```
PROC GLIMMIX DATA = Latency;/*withoutTemp*/
CLASS PondSex FID TankPair Temp TP;
MODEL L = Pondsex|TankPair / DIST = poisson solution LINK = LOG;
Random _Residual_ /Subject = TP;
Run;
quit;
```

Statistical Analysis 4.3 The spawning rate and the fixed effects data analysis with the generalized linear mixed model (GLIMMIX) SAS procedure without the 'PondSex' variable in the model statement was as follows:

```
dm 'log;clear;output;clear';
title1 "test for differences in spawning rate, without pondsex";
options nodate nocenter pageno=1 ls=78 ps=55;
ODS listing;
Data SpawningRate;
Input TP$ FID$ Spawned Temp$ TankPair$ PondSex$;
Datalines;
I       F16     0.33    H       M-F     Mixed
I       F19     0.33    H       M-F     Mixed
I       F21     0.33    H       M-F     Mixed
I       F24     0.33    H       F-F     Mixed
II      F48     0.30    H       F-F     F
II      F43     0.30    H       F-F     F
II      F45     0.30    H       F-F     F
III     F64     0.20    H       F-F     Mixed
III     F70     0.20    H       M-F     Mixed
IV      F76     0.30    H       F-F     F
IV      F79     0.30    H       M-F     F
IV      F84     0.30    H       F-F     F
V       F104    0.27    A       F-F     F
V       F110    0.27    A       M-F     F
V       F100    0.27    A       F-F     F
VI      F112    0.42    A       F-F     Mixed
VI      F113    0.42    A       F-F     Mixed
VI      F114    0.42    A       F-F     Mixed
VI      F116    0.42    A       M-F     Mixed
VI      F117    0.42    A       M-F     Mixed
;
PROC GLIMMIX DATA = SpawningRate;/*without PondSex*/
CLASS PondSex TankPair Temp TP;
MODEL Spawned = TEMP|TankPair / DIST = binomial LINK = LOGIT;
Random _Residual_ /Subject = TP;
Run;
quit;
```

Statistical Analysis 4.4. The spawning rate and the fixed effects data analysis with the generalized linear mixed model (GLIMMIX) SAS procedure without the 'Temperature' variable in the model statement was as follows:

```
dm 'log;clear;output;clear';
title1 "test for differences in spawning rates, without temp";
options nodate nocenter pageno=1 ls=78 ps=55;
ODS listing;
Data SpawningRate;
Input TP$ FID$ Spawned Temp$ TankPair$ PondSex$;
Datalines;
I       F16     0.33    H       M-F     Mixed
I       F19     0.33    H       M-F     Mixed
I       F21     0.33    H       M-F     Mixed
I       F24     0.33    H       F-F     Mixed
II      F48     0.30    H       F-F     F
II      F43     0.30    H       F-F     F
II      F45     0.30    H       F-F     F
III     F64     0.20    H       F-F     Mixed
III     F70     0.20    H       M-F     Mixed
IV      F76     0.30    H       F-F     F
IV      F79     0.30    H       M-F     F
IV      F84     0.30    H       F-F     F
V       F104    0.27    A       F-F     F
V       F110    0.27    A       M-F     F
V       F100    0.27    A       F-F     F
VI      F112    0.42    A       F-F     Mixed
VI      F113    0.42    A       F-F     Mixed
VI      F114    0.42    A       F-F     Mixed
VI      F116    0.42    A       M-F     Mixed
VI      F117    0.42    A       M-F     Mixed
;
PROC GLIMMIX DATA = SpawningRate;/*withoutTemp*/
CLASS PondSex TankPair Temp TP;
MODEL Spawned = Pondsex|TankPair / DIST = binomial LINK = LOGIT;
Random _Residual_ /Subject = TP;
Run;
quit;
```

Statistical Analysis 4.5 The fertilization and the fixed effects data analysis with the generalized linear mixed model (GLIMMIX) SAS procedure without the 'PondSex' variable in the model statement was as follows:

```
dm 'log;clear;output;clear';
title1 "test for difference in fertilization without PondSex";
options nodate nocenter pageno=1 ls=78 ps=55;
ODS listing;
Data Fertilization;
Input TP$ FID$ MID$ Fert SpermUsed$ Temp$ TankPair$ PondSex$;
Datalines;
III  F64  M44  0.60  SC1  H  F-F  Mixed
III  F64  M44  0.70  SC2  H  F-F  Mixed
III  F64  M44  0.60  SC3  H  F-F  Mixed
III  F64  M44  0.70  SC4  H  F-F  Mixed
III  F64  M45  .     SC1  H  F-F  Mixed
III  F64  M45  0.70  SC2  H  F-F  Mixed
III  F64  M45  0.70  SC3  H  F-F  Mixed
III  F64  M45  0.70  SC4  H  F-F  Mixed
III  F64  M46  0.40  SC1  H  F-F  Mixed
III  F64  M46  0.60  SC2  H  F-F  Mixed
III  F64  M46  0.50  SC3  H  F-F  Mixed
III  F64  M46  0.40  SC4  H  F-F  Mixed
III  F70  M44  0.20  SC1  H  M-F  Mixed
III  F70  M44  0.50  SC2  H  M-F  Mixed
III  F70  M44  0.50  SC3  H  M-F  Mixed
III  F70  M44  0.50  SC4  H  M-F  Mixed
III  F70  M45  .     SC1  H  M-F  Mixed
III  F70  M45  0.60  SC2  H  M-F  Mixed
III  F70  M45  0.50  SC3  H  M-F  Mixed
III  F70  M45  0.60  SC4  H  M-F  Mixed
III  F70  M46  0.20  SC1  H  M-F  Mixed
III  F70  M46  0.50  SC2  H  M-F  Mixed
III  F70  M46  0.50  SC3  H  M-F  Mixed
III  F70  M46  0.40  SC4  H  M-F  Mixed
IV   F76  M47  0.30  SC1  H  F-F  F
IV   F76  M47  0.30  SC2  H  F-F  F
IV   F76  M47  0.20  SC3  H  F-F  F
IV   F76  M47  0.40  SC4  H  F-F  F
IV   F76  M48  .     SC1  H  F-F  F
IV   F76  M48  0.40  SC2  H  F-F  F
IV   F76  M48  0.30  SC3  H  F-F  F
IV   F76  M48  0.40  SC4  H  F-F  F
IV   F76  M49  0.20  SC1  H  F-F  F
IV   F76  M49  0.40  SC2  H  F-F  F
IV   F76  M49  0.40  SC3  H  F-F  F
```

IV	F76	M49	.	SC4	H	F-F	F
IV	F79	M47	0.80	SC1	H	M-F	F
IV	F79	M47	0.80	SC2	H	M-F	F
IV	F79	M47	0.80	SC3	H	M-F	F
IV	F79	M47	0.70	SC4	H	M-F	F
IV	F79	M48	.	SC1	H	M-F	F
IV	F79	M48	0.90	SC2	H	M-F	F
IV	F79	M48	0.80	SC3	H	M-F	F
IV	F79	M48	0.70	SC4	H	M-F	F
IV	F79	M49	0.90	SC1	H	M-F	F
IV	F79	M49	0.80	SC2	H	M-F	F
IV	F79	M49	0.70	SC3	H	M-F	F
IV	F79	M49	.	SC4	H	M-F	F
IV	F84	M47	0.60	SC1	H	F-F	F
IV	F84	M47	0.60	SC2	H	F-F	F
IV	F84	M47	0.50	SC3	H	F-F	F
IV	F84	M47	.	SC4	H	F-F	F
IV	F84	M48	.	SC1	H	F-F	F
IV	F84	M48	0.70	SC2	H	F-F	F
IV	F84	M48	0.70	SC3	H	F-F	F
IV	F84	M48	0.90	SC4	H	F-F	F
IV	F84	M49	0.50	SC1	H	F-F	F
IV	F84	M49	0.80	SC2	H	F-F	F
IV	F84	M49	0.20	SC3	H	F-F	F
IV	F84	M49	.	SC4	H	F-F	F
V	F104	M04	0.40	SC1	A	F-F	F
V	F104	M04	0.50	SC2	A	F-F	F
V	F104	M04	0.40	SC3	A	F-F	F
V	F104	M04	0.50	SC4	A	F-F	F
V	F104	M05	0.50	SC1	A	F-F	F
V	F104	M05	0.50	SC2	A	F-F	F
V	F104	M05	0.60	SC3	A	F-F	F
V	F104	M05	0.50	SC4	A	F-F	F
V	F104	M06	0.60	SC1	A	F-F	F
V	F104	M06	0.60	SC2	A	F-F	F
V	F104	M06	0.40	SC3	A	F-F	F
V	F104	M06	0.60	SC4	A	F-F	F
V	F110	M04	0.70	SC1	A	M-F	F
V	F110	M04	0.70	SC2	A	M-F	F
V	F110	M04	0.50	SC3	A	M-F	F
V	F110	M04	0.60	SC4	A	M-F	F
V	F110	M05	0.70	SC1	A	M-F	F
V	F110	M05	0.60	SC2	A	M-F	F
V	F110	M05	0.60	SC3	A	M-F	F
V	F110	M05	0.60	SC4	A	M-F	F
V	F110	M06	0.70	SC1	A	M-F	F

```
V       F110    M06     0.60    SC2     A       M-F     F
V       F110    M06     0.60    SC3     A       M-F     F
V       F110    M06     0.60    SC4     A       M-F     F
V       F100    M04     0.60    SC1     A       F-F     F
V       F100    M04     0.40    SC2     A       F-F     F
V       F100    M04     0.40    SC3     A       F-F     F
V       F100    M04     0.50    SC4     A       F-F     F
V       F100    M05     0.50    SC1     A       F-F     F
V       F100    M05     0.50    SC2     A       F-F     F
V       F100    M05     0.50    SC3     A       F-F     F
V       F100    M05     0.50    SC4     A       F-F     F
V       F100    M06     0.60    SC1     A       F-F     F
V       F100    M06     0.50    SC2     A       F-F     F
V       F100    M06     0.40    SC3     A       F-F     F
V       F100    M06     0.50    SC4     A       F-F     F
;
PROC GLIMMIX DATA = Fertilization;/*without PondSex*/
CLASS PondSex TankPair Temp TP SpermUsed;
MODEL Fert = Temp|TankPair|SpermUsed / DIST = binomial LINK = LOGIT;
Random _Residual_ /Subject = TP;
Run;
quit;
```

Statistical Analysis 4.6. The fertilization and the fixed effects data analysis with the generalized linear mixed model (GLIMMIX) SAS procedure without the 'Temperature' variable in the model statement was as follows:

```
dm 'log;clear;output;clear';
title1 "test for difference in fertilization without temp";
options nodate nocenter pageno=1 ls=78 ps=55;
ODS listing;
Data Fertilization;
Input TP$ FID$ MID$ Fert SpermUsed$ Temp$ TankPair$ PondSex$;
Datalines;
III     F64     M44     0.60    SC1     H       F-F     Mixed
III     F64     M44     0.70    SC2     H       F-F     Mixed
III     F64     M44     0.60    SC3     H       F-F     Mixed
III     F64     M44     0.70    SC4     H       F-F     Mixed
III     F64     M45     .       SC1     H       F-F     Mixed
III     F64     M45     0.70    SC2     H       F-F     Mixed
III     F64     M45     0.70    SC3     H       F-F     Mixed
III     F64     M45     0.70    SC4     H       F-F     Mixed
III     F64     M46     0.40    SC1     H       F-F     Mixed
III     F64     M46     0.60    SC2     H       F-F     Mixed
III     F64     M46     0.50    SC3     H       F-F     Mixed
III     F64     M46     0.40    SC4     H       F-F     Mixed
```

III	F70	M44	0.20	SC1	H	M-F	Mixed
III	F70	M44	0.50	SC2	H	M-F	Mixed
III	F70	M44	0.50	SC3	H	M-F	Mixed
III	F70	M44	0.50	SC4	H	M-F	Mixed
III	F70	M45	.	SC1	H	M-F	Mixed
III	F70	M45	0.60	SC2	H	M-F	Mixed
III	F70	M45	0.50	SC3	H	M-F	Mixed
III	F70	M45	0.60	SC4	H	M-F	Mixed
III	F70	M46	0.20	SC1	H	M-F	Mixed
III	F70	M46	0.50	SC2	H	M-F	Mixed
III	F70	M46	0.50	SC3	H	M-F	Mixed
III	F70	M46	0.40	SC4	H	M-F	Mixed
IV	F76	M47	0.30	SC1	H	F-F	F
IV	F76	M47	0.30	SC2	H	F-F	F
IV	F76	M47	0.20	SC3	H	F-F	F
IV	F76	M47	0.40	SC4	H	F-F	F
IV	F76	M48	.	SC1	H	F-F	F
IV	F76	M48	0.40	SC2	H	F-F	F
IV	F76	M48	0.30	SC3	H	F-F	F
IV	F76	M48	0.40	SC4	H	F-F	F
IV	F76	M49	0.20	SC1	H	F-F	F
IV	F76	M49	0.40	SC2	H	F-F	F
IV	F76	M49	0.40	SC3	H	F-F	F
IV	F76	M49	.	SC4	H	F-F	F
IV	F79	M47	0.80	SC1	H	M-F	F
IV	F79	M47	0.80	SC2	H	M-F	F
IV	F79	M47	0.80	SC3	H	M-F	F
IV	F79	M47	0.70	SC4	H	M-F	F
IV	F79	M48	.	SC1	H	M-F	F
IV	F79	M48	0.90	SC2	H	M-F	F
IV	F79	M48	0.80	SC3	H	M-F	F
IV	F79	M48	0.70	SC4	H	M-F	F
IV	F79	M49	0.90	SC1	H	M-F	F
IV	F79	M49	0.80	SC2	H	M-F	F
IV	F79	M49	0.70	SC3	H	M-F	F
IV	F79	M49	.	SC4	H	M-F	F
IV	F84	M47	0.60	SC1	H	F-F	F
IV	F84	M47	0.60	SC2	H	F-F	F
IV	F84	M47	0.50	SC3	H	F-F	F
IV	F84	M47	.	SC4	H	F-F	F
IV	F84	M48	.	SC1	H	F-F	F
IV	F84	M48	0.70	SC2	H	F-F	F
IV	F84	M48	0.70	SC3	H	F-F	F
IV	F84	M48	0.90	SC4	H	F-F	F
IV	F84	M49	0.50	SC1	H	F-F	F
IV	F84	M49	0.80	SC2	H	F-F	F

```
IV      F84     M49     0.20    SC3     H       F-F     F
IV      F84     M49     .       SC4     H       F-F     F
V       F104    M04     0.40    SC1     A       F-F     F
V       F104    M04     0.50    SC2     A       F-F     F
V       F104    M04     0.40    SC3     A       F-F     F
V       F104    M04     0.50    SC4     A       F-F     F
V       F104    M05     0.50    SC1     A       F-F     F
V       F104    M05     0.50    SC2     A       F-F     F
V       F104    M05     0.60    SC3     A       F-F     F
V       F104    M05     0.50    SC4     A       F-F     F
V       F104    M06     0.60    SC1     A       F-F     F
V       F104    M06     0.60    SC2     A       F-F     F
V       F104    M06     0.40    SC3     A       F-F     F
V       F104    M06     0.60    SC4     A       F-F     F
V       F110    M04     0.70    SC1     A       M-F     F
V       F110    M04     0.70    SC2     A       M-F     F
V       F110    M04     0.50    SC3     A       M-F     F
V       F110    M04     0.60    SC4     A       M-F     F
V       F110    M05     0.70    SC1     A       M-F     F
V       F110    M05     0.60    SC2     A       M-F     F
V       F110    M05     0.60    SC3     A       M-F     F
V       F110    M05     0.60    SC4     A       M-F     F
V       F110    M06     0.70    SC1     A       M-F     F
V       F110    M06     0.60    SC2     A       M-F     F
V       F110    M06     0.60    SC3     A       M-F     F
V       F110    M06     0.60    SC4     A       M-F     F
V       F100    M04     0.60    SC1     A       F-F     F
V       F100    M04     0.40    SC2     A       F-F     F
V       F100    M04     0.40    SC3     A       F-F     F
V       F100    M04     0.50    SC4     A       F-F     F
V       F100    M05     0.50    SC1     A       F-F     F
V       F100    M05     0.50    SC2     A       F-F     F
V       F100    M05     0.50    SC3     A       F-F     F
V       F100    M05     0.50    SC4     A       F-F     F
V       F100    M06     0.60    SC1     A       F-F     F
V       F100    M06     0.50    SC2     A       F-F     F
V       F100    M06     0.40    SC3     A       F-F     F
V       F100    M06     0.50    SC4     A       F-F     F
;
PROC GLIMMIX DATA = Fertilization;/*withoutTemp*/
CLASS PondSex TankPair Temp TP SpermUsed;
MODEL Fert = Pondsex|TankPair|SpermUsed / DIST = binomial LINK = LOG;
Random _Residual_ /Subject = TP;
Run;
quit;
```

Statistical Analysis 4.7. The males used and the fixed effects data analysis with the generalized linear mixed model (GLIMMIX) SAS procedure without the 'Temperature' variable in the model statement was as follows:

```
dm 'log;clear;output;clear';
title1 "test for differences in males used in fertilization";
options nodate nocenter pageno=1 ls=78 ps=55;
ODS listing;
Data Males;
Input TP$ FID$ MID$ Fert;
Datalines;
III     F64     M44     0.6
III     F64     M44     0.7
III     F64     M44     0.6
III     F64     M44     0.7
III     F64     M45     .
III     F64     M45     0.7
III     F64     M45     0.7
III     F64     M45     0.7
III     F64     M46     0.4
III     F64     M46     0.6
III     F64     M46     0.5
III     F64     M46     0.4
III     F70     M44     0.2
III     F70     M44     0.5
III     F70     M44     0.5
III     F70     M44     0.5
III     F70     M45     .
III     F70     M45     0.6
III     F70     M45     0.5
III     F70     M45     0.6
III     F70     M46     0.2
III     F70     M46     0.5
III     F70     M46     0.5
III     F70     M46     0.4
IV      F76     M47     0.3
IV      F76     M47     0.3
IV      F76     M47     0.2
IV      F76     M47     0.4
IV      F76     M48     .
IV      F76     M48     0.4
IV      F76     M48     0.3
IV      F76     M48     0.4
IV      F76     M49     0.2
IV      F76     M49     0.4
IV      F76     M49     0.4
```

IV	F76	M49	.
IV	F79	M47	0.8
IV	F79	M47	0.8
IV	F79	M47	0.8
IV	F79	M47	0.7
IV	F79	M48	.
IV	F79	M48	0.9
IV	F79	M48	0.8
IV	F79	M48	0.7
IV	F79	M49	0.9
IV	F79	M49	0.8
IV	F79	M49	0.7
IV	F79	M49	.
IV	F84	M47	0.6
IV	F84	M47	0.6
IV	F84	M47	0.5
IV	F84	M47	.
IV	F84	M48	.
IV	F84	M48	0.7
IV	F84	M48	0.7
IV	F84	M48	0.9
IV	F84	M49	0.5
IV	F84	M49	0.8
IV	F84	M49	0.2
IV	F84	M49	.
V	F104	M04	0.4
V	F104	M04	0.5
V	F104	M04	0.4
V	F104	M04	0.5
V	F104	M05	0.5
V	F104	M05	0.5
V	F104	M05	0.6
V	F104	M05	0.5
V	F104	M06	0.6
V	F104	M06	0.6
V	F104	M06	0.4
V	F104	M06	0.6
V	F110	M04	0.7
V	F110	M04	0.7
V	F110	M04	0.5
V	F110	M04	0.6
V	F110	M05	0.7
V	F110	M05	0.6
V	F110	M05	0.6
V	F110	M05	0.6
V	F110	M06	0.7

```
V       F110    M06     0.6
V       F110    M06     0.6
V       F110    M06     0.6
V       F100    M04     0.6
V       F100    M04     0.4
V       F100    M04     0.4
V       F100    M04     0.5
V       F100    M05     0.5
V       F100    M05     0.5
V       F100    M05     0.5
V       F100    M05     0.5
V       F100    M06     0.6
V       F100    M06     0.5
V       F100    M06     0.4
V       F100    M06     0.5
;
PROC GLIMMIX DATA = Males;
CLASS TP MID;
MODEL Fert = MID/ DIST = binomial LINK = LOG;
Random _Residual_ /group = TP;
Run;
quit;
```

Data and statistics used for Chapter 5. Statistical Analysis 5.1 tested the effect of spawning trials on fertilization estimates. Statistical Analysis 5.2 tested the effect of expected outcome, year, and the interaction of the expected outcome and year on the observed outcomes, and it was used to generate the error matrix.

Statistical Analysis 5.1. Data analysis using the generalized linear mixed model (GLIMMIX) SAS procedure was as follows:

```
dm 'log;clear;output;clear';
title1 "test for the effects of 10 spawning trials on fertilization";
options nodate nocenter pageno=1 ls=78 ps=55;
ODS listing;
Data Fertilization;
Input T$ FID$ F;
Datalines;
I 08F64      .
I 08F65      .
I 08F67      .
I 08F43      0.11
I 08F44      0.18
I 08F46      0.14
I 08F51      0.00
I 08F69      0.00
II 08F118    .
II 08F104    0.00
III 08F140   .
III 08F142   .
III 08F143   .
III 08F144   .
III 08F145   .
III 08F123   0.00
III 08F128   0.00
III 08F147   .
III 08F124   0.15
III 08F125   0.20
III 08F126   0.18
III 08F146   .
IV 09F33     0.02
IV 09F61     .
IV 09F64     .
IV 09F37     .
IV 09F42     0.18
IV 09F57     .
IV 09F60     .
IV 09F28     0.00
```

IV 09F30	0.00
IV 09F36	.
IV 09F38	0.21
IV 09F40	0.00
IV 09F41	0.25
IV 09F43	0.26
IV 09F59	.
IV 09F58	.
V 09F73	0.26
V 09F65	0.03
V 09F68	0.26
V 09F69	0.00
V 09F70	.
V 09F72	.
V 09F66	0.26
V 09F71	.
VI 09F86	0.40
VI 09F94	.
VI 09F95	0.01
VI 09F96	.
VI 09F115	.
VI 09F116	.
VI 09F85	0.00
VI 09F87	0.16
VI 09F88	0.13
VI 09F89	0.28
VI 09F91	0.04
VI 09F93	0.06
VI 09F98	0.28
VI 09F99	0.40
VI 09F100	.
VI 09F101	0.00
VI 09F102	.
VI 09F103	.
VI 09F104	.
VI 09F105	.
VI 09F106	.
VI 09F107	.
VI 09F108	.
VI 09F109	.
VI 09F111	.
VI 09F112	.
VI 09F113	.
VI 09F97	0.43
VI 09F110	.
VII 09F128	.

VII 09F131	.
VII 09F133	.
VII 09F118	0.12
VII 09F119	0.58
VII 09F120	0.28
VII 09F121	0.18
VII 09F122	0.38
VII 09F123	0.18
VII 09F124	.
VII 09F125	.
VII 09F126	.
VII 09F127	.
VII 09F129	.
VII 09F130	.
VII 09F132	.
VII 09F134	.
VII 09F135	.
VIII 10F11	0.00
VIII 10F16	.
VIII 10F18	.
VIII 10F02	0.85
VIII 10F03	.
VIII 10F06	0.00
VIII 10F10	0.06
VIII 10F20	.
VIII 10F22	.
VIII 10F04	0.78
VIII 10F05	0.83
VIII 10F07	0.17
VIII 10F08	0.17
VIII 10F12	0.00
VIII 10F21	.
VIII 10F24	.
VIII 10F01	.
VIII 10F09	0.46
VIII 10F13	.
VIII 10F14	.
VIII 10F15	.
VIII 10F17	.
VIII 10F19	.
VIII 10F23	.
IX 10F31	0.08
IX 10F34	0.01
IX 10F43	.
IX 10F28	0.00
IX 10F30	0.01

IX 10F32	0.00
IX 10F36	0.13
IX 10F39	.
IX 10F40	.
IX 10F41	.
IX 10F25	0.20
IX 10F26	0.13
IX 10F29	0.03
IX 10F33	0.01
IX 10F35	0.01
IX 10F37	0.32
IX 10F45	.
IX 10F38	.
IX 10F42	.
IX 10F44	.
IX 10F27	0.06
X 10F50	0.16
X 10F72	.
X 10F75	.
X 10F53	0.26
X 10F54	0.25
X 10F59	0.23
X 10F61	0.01
X 10F65	.
X 10F66	.
X 10F67	.
X 10F68	.
X 10F69	.
X 10F71	.
X 10F79	.
X 10F46	0.26
X 10F47	0.44
X 10F48	0.00
X 10F49	0.22
X 10F52	0.16
X 10F55	0.39
X 10F57	0.03
X 10F58	0.10
X 10F60	0.37
X 10F62	0.53
X 10F63	0.32
X 10F64	0.02
X 10F70	.
X 10F73	.
X 10F74	.
X 10F76	.

```
X 10F77     .
X 10F78     .
X 10F51     0.00
X 10F56     0.37
;
PROC GLIMMIX DATA = Fertilization;
CLASS T FID;
MODEL F = T / DIST = binomial LINK =logit;
Run;
quit;
```

Statistical Analysis 5.2. Data analysis using the generalized linear mixed model (GLIMMIX) SAS procedure was as follows:

```
data sevencat;
input year exp_outcome obs_outcome trial count;
datalines;
2008 1 1     1     0
2008 2 2     1     0
2008 3 3     1     3
2008 4 4     1     0
2008 5 5     1     0
2008 6 6     1     0
2008 7 7     1     0
2008 1 1     2     1
2008 2 2     2     0
2008 3 3     2     1
2008 4 4     2     0
2008 5 5     2     0
2008 6 6     2     1
2008 7 7     2     0
2008 1 1     3     0
2008 2 2     3     1
2008 3 3     3     5
2008 4 4     3     0
2008 5 5     3     3
2008 6 6     3     0
2008 7 7     3     0
2009 1 1     1     0
2009 2 2     1     0
2009 3 3     1     3
2009 4 4     1     1
2009 5 5     1     3
2009 6 6     1     1
2009 7 7     1     0
2009 1 1     2     0
```

```
2009 2 2        2       1
2009 3 3        2       0
2009 4 4        2       1
2009 5 5        2       1
2009 6 6        2       0
2009 7 7        2       1
2009 1 1        3       0
2009 2 2        3       0
2009 3 3        3       0
2009 4 4        3       0
2009 5 5        3       0
2009 6 6        3       3
2009 7 7        3       6
2010 1 1        1       0
2010 2 2        1       0
2010 3 3        1       2
2010 4 4        1       3
2010 5 5        1       4
2010 6 6        1       0
2010 7 7        1       7
2010 1 1        2       0
2010 2 2        2       0
2010 3 3        2       2
2010 4 4        2       1
2010 5 5        2       3
2010 6 6        2       3
2010 7 7        2       3
2010 1 1        3       0
2010 2 2        3       0
2010 3 3        3       2
2010 4 4        3       3
2010 5 5        3       9
2010 6 6        3       0
2010 7 7        3       1
;
run;
proc glimmix data = sevencat;
class obs_outcome exp_outcome trial year;
model obs_outcome = exp_outcome year exp_outcome*year/ link = clogit dist = multinomial
solution;
weight count;
output out = year200820092010 pred(ilink)=model_predicted;
run;
proc sort data = year200820092010; by exp_outcome _Level_  ;
proc means noprint data = year200820092010; by exp_outcome _Level_  ;
var model_predicted;
```

```
output out = confusion200820092010 mean = probability_correct;
run
;
```

Data and Statistics used for Chapter 7.

Statistical Analysis 7.1 tested the relationship of location to ovarian development for channel catfish conditioned at ambient temperatures and assessed using ultrasound imaging index in Louisiana (LA) and in Arkansas (ARK).

```
options pageno=1 linesize=80;
goptions reset=all;
title "Channel catfish ovarian development in ponds in LA (LSUAC-ARS)and ARK (BLCH)";
data LouisianaArkansas;
        input stage$ location$ frequency;
        datalines;
4   LA1 167
5   LA1  148
4   ARK1  20
5   ARK1  12
4 LA2 92
5 LA2 88
4 ARK2 96
5 ARK2 145
;
run;
* Print data set;
proc print data=LouisianaArkansas;
run;
* Logistic regression;
proc logistic data=LouisianaArkansas;
        class stage location;
        model stage = location / lackfit;
        freq frequency;
run;
* For comparison, run tests of independence;
proc freq data=LouisianaArkansas;
        tables stage*location / chisq cellchi2 expected out=percents outpct;
        weight frequency;
        * Can compute an exact test if frequencies are low;
        * Not recommended for large data sets;
run;
* Print output data file containing percents;
proc print data=percents;
run;
* Generate bar chart showing percentages;
```

```
proc gchart data=percents;
        vbar location / sumvar=pct_col subgroup=stage;
run;
quit;
```

Statistical Analysis 7.2 tested the relationship of egg quality to ultrasound imaging assessments Categories 4 and 5 of three batches of fish selected for analysis because they shared similar conditions of ultrasound imaging assessments, transportation, and dates of artificial spawning.

```
dm 'log;clear;output;clear';
title1 "Test for difference in quality of eggs of ultrasound assessments Category 4 and 5";
options nodate nocenter pageno=1 ls=78 ps=55;
ODS listing;
Data proportions;
input Category$ outcome$ count;
datalines;
4&5    Low    20
4&5    High   5
5      Low    40
5      High   16
4      Low    26
4      High   18
;
Proc freq data = proportions; weight count;
Table Category*outcome/ fisher;
run;
quit;
```

Statistical Analysis 7.3 tested the relationship of egg quality to two types of ovarian assessments – ultrasound imaging assessments of internal morphology and external morphology assessments commonly used in commercial hatcheries.

```
dm 'log;clear;output;clear';
title1 "test for difference in proportion of quality of eggs of two types of assessments -
ultrasound assessments Category 4 and 5 (UA) and farm external morphology assessments
Category 3, 4 and 5 (FA)";
options nodate nocenter pageno=1 ls=78 ps=55;
ODS listing;
Data proportions;
input AssessmentType$ outcome$ count;
datalines;
UA     Low    86
UA     High   39
FA     Low    29
FA     High   4
;
```

```
Proc freq data = proportions; weight count;
Table AssessmentType*outcome/ fisher;
run;
quit;
```

Category Equivalency Tables. The equivalency table developed for standardization of the raw data on ultrasound imaging assessments during 2008 was as follows:

Table B.1 The equivalency table was used for standardization of the second set of ultrasound imaging sampling dates during 2008 of channel catfish ovarian development in Chapter 7. Ponds B4 (0.3 ha) and B6 (0.3 ha) were at the Louisiana State University Agricultural Center, Aquaculture Research Station in Baton Rouge, Louisiana. Pond 1 (0.4 ha) was at Baxter Lands Company hatchery in Arkansas City, Arkansas.

Categories	Apr 25	Apr 28	Apr 29	May 3	Equivalency
Pond Sampled	B6	B6	B4	Pond 1	
1. Undeveloped	6	40	6	0	1. Undeveloped
2. Under-developed	6	65	13	0	2. Under-developed
3. Developing	8	176	45	9	3. Developing
3.5	9	151	43	19	3. Developing
4. Advanced	8	109	58	12	4. Advanced
4.5	2	0	0	8	4. Advanced
5. Mature	10	95	51	12	5. Mature
6. Spawned	0	0	5	0	6. Spawned
7. Atretic	0	0	1	0	7. Atretic

Table B.2 The equivalency table was used for standardization of the second set of ultrasound imaging sampling dates during 2008 of channel catfish ovarian development in Chapter 7. Ponds B4 (0.3 ha) and B6 (0.3 ha) were at the Louisiana State University Agricultural Center, Aquaculture Research Station in Baton Rouge, Louisiana. Pond 1 (0.4 ha), Pond 41 and Pond 3 were at Baxter Lands Company hatchery in Arkansas City, Arkansas.

Raw Data Assignment	May 12	May 12	May 16	May 17	May 18	Equivalency
Pond Sampled	B4	B6	Pond 1	Pond 41*	Pond 3*	
1. Undeveloped	40	80	0	0	0	1. Undeveloped
2. Under-developed	14	65	0	3	0	2. Under-developed
3. Developing	37	102	6	14	0	3. Developing
3.5	24	70	29	31	2	3. Developing
4. Advanced	38	54	12	17	11	4. Advanced
4.5	0	0	8	27	21	4. Advanced
5. Mature	49	39	24	39	82	5. Mature
6. Spawned	0	16	0	0	0	6. Spawned
7. Atretic	0	10	0	0	2	7. Atretic

*Data on pond size not available.

Appendix C*
Ultrasonographic Monitoring of Channel Catfish Ovarian Development

Gonadal development throughout the life cycle of channel catfish *Ictalurus punctatus* from karyogamy during fertilization, through gonadal differentiation, juvenility, and reproductive activity in adults is controlled by genetic factors (Tiersch et al. 1992; Wolters and Tiersch 2004), and the endocrine system in concert with the environment (Silverstein and Small 2004). Ovarian development in mature channel catfish and the physiological processes directing it are directly affected by oocyte development, starting from recruitment of oogonia (12–15 µm in diameter), transitioning to pre-vitellogenic (15–240 µm), and vacuolated (240–650 µm) and vitellogenic (650–3,000 µm) oocytes. These become secondary oocytes, complete the first meiotic division, undergo meiotic arrest at metaphase of the second meiotic division, and are ready to be ovulated and fertilized (Grizzle 1985). Oocyte development and maturation for spawning in channel catfish begins at 2 to 3 yr of age, although most producers use 3-yr-old catfish for induced spawning (Barrero et al. 2007; Barrero et al. 2008). Overall, ovarian and oocyte development is complex, involving environmental, hormonal, cellular, and molecular processes leading to ovulation. Various reproductive indices exist for interpreting these interdependent processes, and for assessing the state of ovarian maturity to select channel catfish for induced spawning. These reproductive indices are obtained using invasive and non-invasive methods.

---

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Invasive methods for evaluating ovarian and oocyte development in channel catfish populations have included ovarian catheterization (Markmann and Doroshov 1983), germinal vesicle visualization (Stoeckel 2000), monitoring of serum hormonal profiles, gross examination of the ovary and oocytes, measurement of gonadosomatic index (gonad weight/body weight x 100) (Brauhn and McCraren 1975), and preservation of ovaries for histological analysis (MacKenzie et al. 1989). The most commonly used non-invasive method for assessing channel catfish ovarian development is visual examination of external morphology, which includes monitoring for a soft, rounded, distended abdomen extending past the pelvic fin and a swollen, reddish urogenital orifice (Clemens and Sneed 1957). Other non-invasive methods for identifying reproductive females are direct observation of active spawning behavior (Bates and Tiersch 1998; Phelps et al. 2007; Lang and Tiersch 2007), and measurement of thermal exposure (degree-days) for prediction of spawning in ponds (Pawiroredjo et al. 2008).

Ultrasonography, a non-invasive technology, has been used in as many as 19 fish species for sex identification (Matsubara et al. 1999; Colombo et al. 2004; Wildhaber et al. 2005), and for development of reproductive indices such as cross-sectional ovarian and testes diameter, gonad volume, and egg diameter (Bryan et al. 2007; Wildhaber et al. 2007; Newman et al. 2008) (Table C.1). Two catfishes, the Neosho madtom *Noturus placidus* and the African catfish *Clarias gariepinus,* were studied for monitoring the ovarian reproductive condition before and during the natural spawning season (Bryan et al. 2005; Laszlo et al. 2008).

Table C.1 The species (n = 19) and references (n = 23) on use of ultrasonography in fish reproduction were listed. These fishes were grouped below into two main categories (i) freshwater, and (ii) marine and anadromous, according to family, genus and species, with corresponding citations. It was possible to view ovaries and testes in 85% of the species listed.

Common name*	Scientific name*	Citation
Freshwater		
Stellate sturgeon	*Acipenser stellatus*	Moghim et al. 2002
Shovelnose sturgeon	*Scaphirhynchus platorynchus*	Colombo et al. 2004, Wildhaber et al. 2005, 2007, Bryan et al. 2007
Pallid sturgeon	*Scaphirhynchus albus*	Wildhaber et al. 2005, Bryan et al. 2007
Neosho madtom	*Noturus placidus*	Bryan et al. 2005
African catfish	*Clarias gariepinus*	Lazlo et al. 2008
Murray cod	*Maccullochella peelii*	Newman et al. 2008
Marine/Anadromous		
Pacific herring	*Clupea pallasi*	Bonar et al. 1989
Atlantic cod	*Gadus morhua*	Karlsen and Holm 1994
Barfin flounder	*Verasper moseri*	Matsubara et al. 1999
Atlantic halibut	*Hippoglossus hippoglossus*	Shields et al. 1993, Martin-Robichaud and Rommens 2001
Winter flounder	*Pseudopleuronectes americanus*	Martin-Robichaud and Rommens 2001
Yellowtail flounder	*Limanda ferruginea*	Martin-Robichaud and Rommens 2001
Haddock	*Melanogrammus aeglefinus*	Martin-Robichaud and Rommens 2004
Atlantic salmon	*Salmo salar*	Mattson 1991
Coho salmon	*Oncorhynchus kisutch*	Martin et al. 1983
Rainbow trout	*Oncorhynchus mykiss*	Evans et al. 2004a,b
Striped bass	*Morone saxatilis*	Blythe et al. 1994, Will et al. 2002, Jennings et al. 2005
Red hind	*Epinephelus guttatus*	Whiteman et al. 2005
Broadnose sevengill shark	*Notorynchus cepedianus*	Daly et al. 2007

*According to Nelson et al. 2004.

Although ultrasonography has been used to estimate fillet yield in channel catfish (Bosworth et al. 2001), no ultrasound imaging procedures exist for viewing the ovary of channel catfish. This chapter describes ultrasound imaging of ovaries of channel catfish at different stages of gonadal development, and corresponding histological profiles.

Ultrasound Procedures for Viewing of Channel Catfish Ovaries. The natural spawning season for channel catfish in Baton Rouge, Louisiana (Latitude 30° 32’ 24” N, 91° 5’ 24” W) typically starts in mid-late April when ambient water temperatures remain within a range (21–30 C) conducive to spawning (Lang et al. 2003; Pawiroredjo et al. 2008), and continues through May and sometimes into July (Bates and Tiersch 1998). Adult (3–4 yr old) female channel catfish were held in 0.40-ha ponds with blower-driven aeration, and were sampled during early, middle, and late periods of the natural spawning season in 2008. Fish were captured by seining the ponds, and were held in concrete raceways at a salinity of 5 ppt (solar salt, Cargill Inc. Minneapolis, Minnesota, USA) to reduce osmotic imbalances due to stress for 1-3 d before ultrasound imaging and ovary sample collection for histological processing.

Channel catfish were caught with polyethylene dip nets from the concrete raceways, and placed in a portable, 49-L cooler (Sportsman™ 52 Quart, Igloo Products Corp., Katy, TX, USA) filled with water. The fish maintained an upright swimming position (ventral recumbency), while the left side was scanned anterior to the base of the pelvic fin, and posterior to the dorsal fin (Figure C.1). B-Mode ultrasound images were obtained using a laptop-computer ultrasound unit, the Classic TelaVet 1000™ Veterinary Digital Ultrasound Module (Telemed UAB, Vilnius, Lithuania) with a multi-frequency (5–8 MHz) linear probe (model LV7.5/60/96Z) set at 8 MHz.

During the entire procedure, the probe and the catfish were completely submersed in water, which provided the sole ultrasound transmission medium. The location of the ovary and the largest cross-section were determined by scanning the left side of the abdomen between the pectoral and dorsal fins with the probe, with the probe tip aligned to the bottom (ventral) side of the fish, and the cable end of the probe aligned to the top (dorsal) aspect of the fish. Ultrasound images were recorded on the hard drive of the laptop computer, with each image labeled with the corresponding identification number (i.e., Floy tag) of the fish.

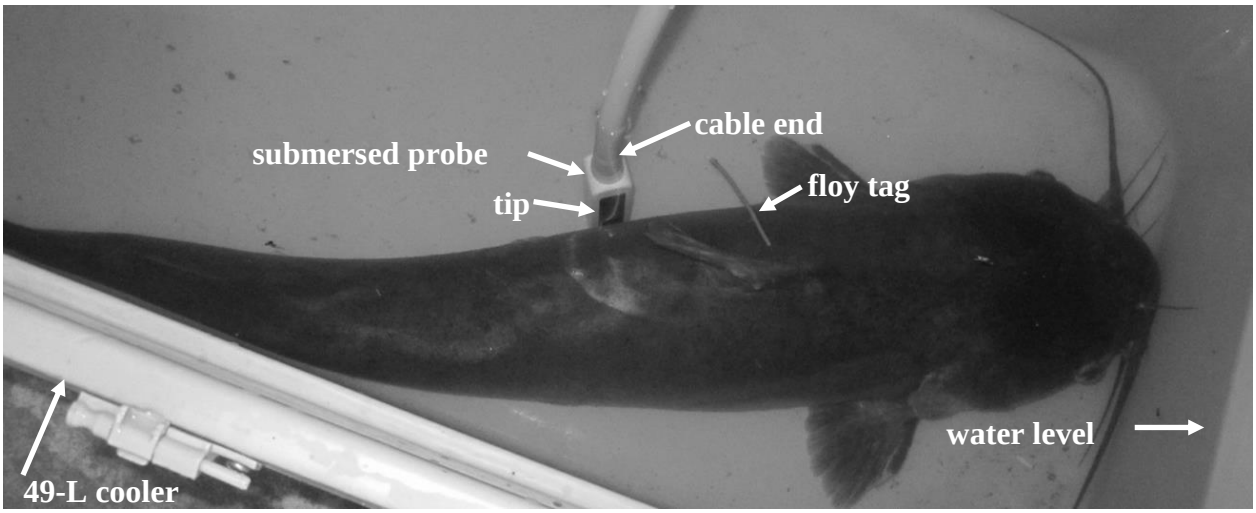


Figure C.1. For viewing, the channel catfish were completely submerged in water, with the ultrasound probe placed on the left side, the cable end (connected to the ultrasound unit) located dorsally (adjacent to the spine), and the probe tip located ventrally (adjacent to bottom of the abdomen).

B-Mode ultrasound images of the ovary were created with the emission of ultrasound waves (in this case, 8 million cycles of ultrasound waves per sec) by piezoelectric crystal elements inside the linear array probe. These ultrasound waves were transmitted into the water, which acted as a transmission medium (a similar function is served by application of ultrasound gel to eliminate the air interface between the probe and the surface of the anatomy being scanned). The emitted acoustic waves made contact first with the skin, and subsequently with

muscle and ovarian tissues in the area at which the probe was positioned (Figure C.1). The return of these ultrasound waves to the probe was displayed in a rectangular, two dimensional gray-scale image on the laptop monitor. The ultrasound echoes were recorded as dots along a vertical axis, with the dots located in the near view field of the image (top of the display image) creating ultrasound images of anatomical structures (skin and muscle) closest to the probe, and the dots in the far view field of the image (bottom of the display), creating ultrasound images of anatomical structures (ovarian structure) furthest from the probe (Figure C.2).

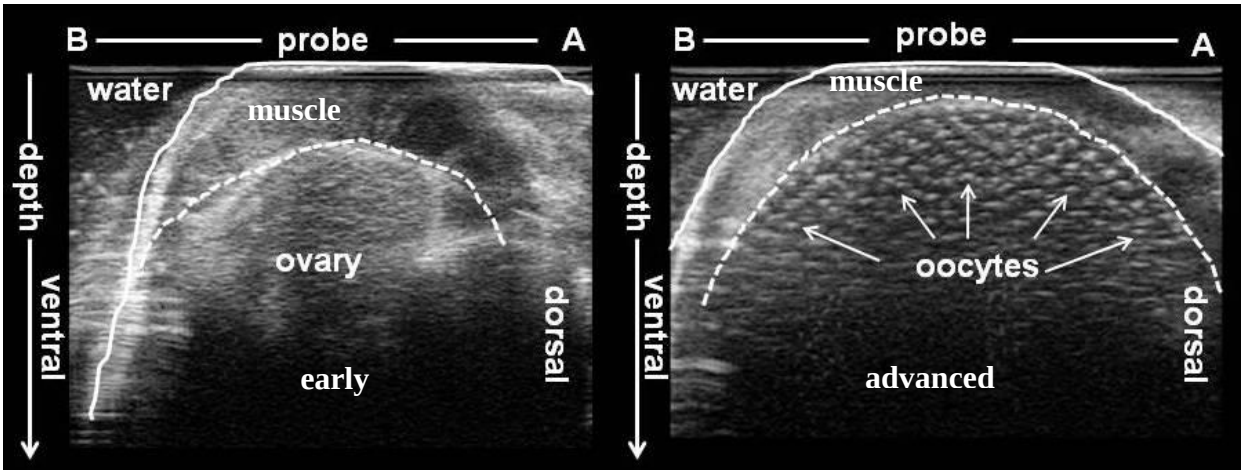


Figure C.2 The image produced by a linear array probe is a rectangular, gray-scale ultrasound image, with the top of the image corresponding to the position of the probe. The cable end of the probe (A) corresponds to the top of the fish (right side of the image), and the tip of the probe (B) corresponds to the bottom of the fish (left side of the image). The top of the image (near field) corresponds to anatomical structures closest to the probe, with the skin depicted by the outermost solid line delineating the curving external perimeter of the fish, followed by muscle tissue, the ovary (dashed curved line), and oocytes (indicated by arrows). The image on the left represents early ovarian development in channel catfish, and the image on the right a more advanced stage.

The position of the dots along the vertical axis of the ultrasound image display represents the depth (mm) of the internal anatomical structures from which the echo originated. The brightness of the dot is proportional to the strength of the returning echo, and corresponds to an intensity within a 256 gray-scale range, with the brighter grays representing echoes of greater intensity. These vertical axis lines, when aligned, represent parallel scan lines produced by acoustic pulses and echoes at different points on the linear array of elements, which are rectangular in shape,

arranged in a straight line, and produce a cross-sectional gray-scale image of the tranverse scanning plane of the ovary.

Thus, one of the key elements in interpreting the ultrasound image irrespective of gonadal condition of the fish is to understand the relationship of the physical position of the probe on the external anatomy of the channel catfish (Figure C.1), and the corresponding probe and anatomical structures in the resulting display image (Figure C.2). When the probe was placed on

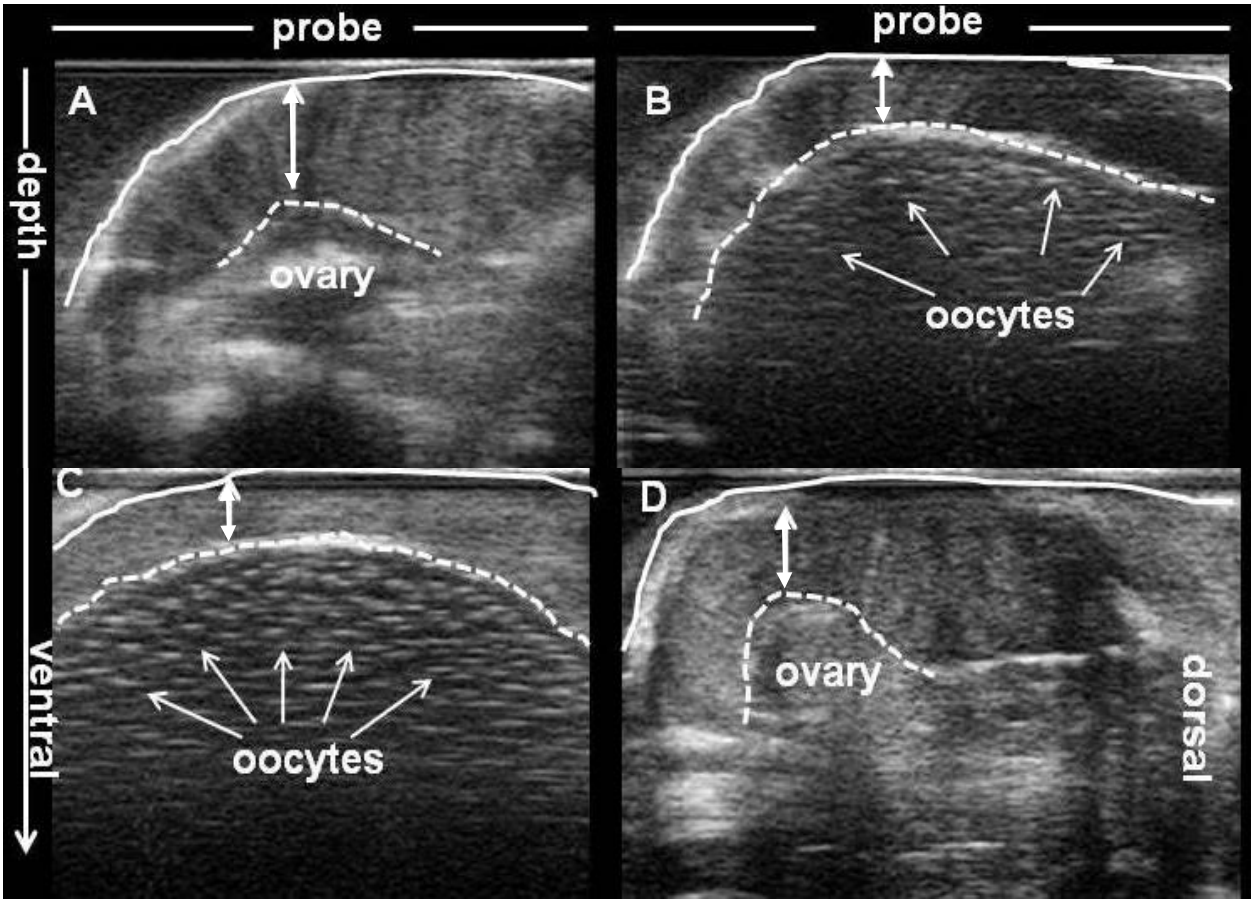


Figure C.3 Ultrasonography provided direct images of channel catfish ovaries during the natural spawning season of channel catfish, revealing distinct gonadal appearances for early (A), developing (B, C) and atretic (D) ovaries. The curved solid white line depicts the skin, the anatomical structure closest to the probe. The dashed line depicts the contour of the ovary, with the dorsal and ventral aspect of the fish on the right and left side of each image, and the double-headed arrows between the skin and the ovary showing the changes in thickness of the body wall in each image. Oocytes appear flattened rather than rounded probably due to the polar distributions of aqueous and lipid compartments

the lateral aspect of the abdomen (Figure C.1), the orientation of the ultrasound image in the monitor was displayed with the top of the image (the near field view) representing the nearest distance to the probe, and with the bottom of the image (the far field view) representing the furthest distance from the probe. In these ultrasound images, the cable end corresponded to the right side of the image (dorsal aspect of the fish), and the tip of the probe corresponded to the left side of the image (ventral aspect of the fish) (Figure C.2), but this orientation of the fish anatomy can be switched by using the ultrasound software controls such that the left side of the image corresponds to the dorsal aspect of the fish and the right side of the image corresponds to the ventral side of the fish. The relationship of the orientation of the probe with respect to the external anatomical positioning of the probe and the internal anatomy of the fish should be clearly defined for basic interpretation of ultrasound images (Figure C.3).

Early gonadal development (Figure C.3A) was seen with more frequency early in the natural spawning season (i.e., early April), with ultrasound images displaying a small ovarian size. At this time the shape of the ovary was frequently not clearly defined, or not clearly distinguished from surrounding internal structures, and there were no visible oocytes. The distance of the abdominal muscle between the periphery of the ovary and the skin (the body wall) was at its widest point.

Ovarian growth was noticeable during the middle and late period of the natural spawning season (i.e., late April to early July), with ultrasound images displaying a progressively enlarging ovarian size (Figure C.3B and C). The ovarian structure was immediately visible and did not require multiple abdominal scans to be identified. The shape of the ovary was clearly visible,

with the outermost periphery of the ovary curved and clearly defined, similar to the skin margin of the fish which was always visible during ultrasound scans. Oocytes were immediately visible during ultrasound scans. Individual oocytes and a high degree of organization within the ovary were discernible as the spawning season and gonadal growth advanced. The thickness of the body wall between the periphery of the ovary and the skin progressively narrowed with increased ovarian growth.

Towards the end of the spawning season (i.e., July), ultrasound images revealed gonads that were drastically reduced in size, and undergoing atresia (Figure C.3D). Atretic ovaries could be identified by images displaying a small, disfigured ovarian wall, and disorganized, disintegrating oocytes that lacked a clear perimeter and regular shape. The body wall thickened with the reduced size of ovaries undergoing atresia.

Histological Profiles of Ultrasound Images. Fish were placed into a lethal dose of MS-222 for removal of ovaries, which were preserved in 10% neutral buffered formalin (NBF). After storage ($\geq$ 1 month), the ovaries were sectioned through the largest cross-section, corresponding to the position of the ultrasound probe, and sent for histological processing to the Histology Laboratory of the Louisiana State University School of Veterinary Medicine, Baton Rouge, Louisiana. The section widths of the ovary samples on the histology slides were 3 – 4 µm, and the chemical stains used were hematoxylin and eosin. Digital images of histology slides were obtained using a stereoscope (Nikon SMZ-U, Tokyo, Japan). A mm-increment ruler was positioned in the upper left corner of each histology slide to provide a standard size reference.

Histology profiles (Figure C.4) corresponding to the ultrasound images collected during the spawning season (Figure C.3) revealed the microscopic biological progression of ovarian

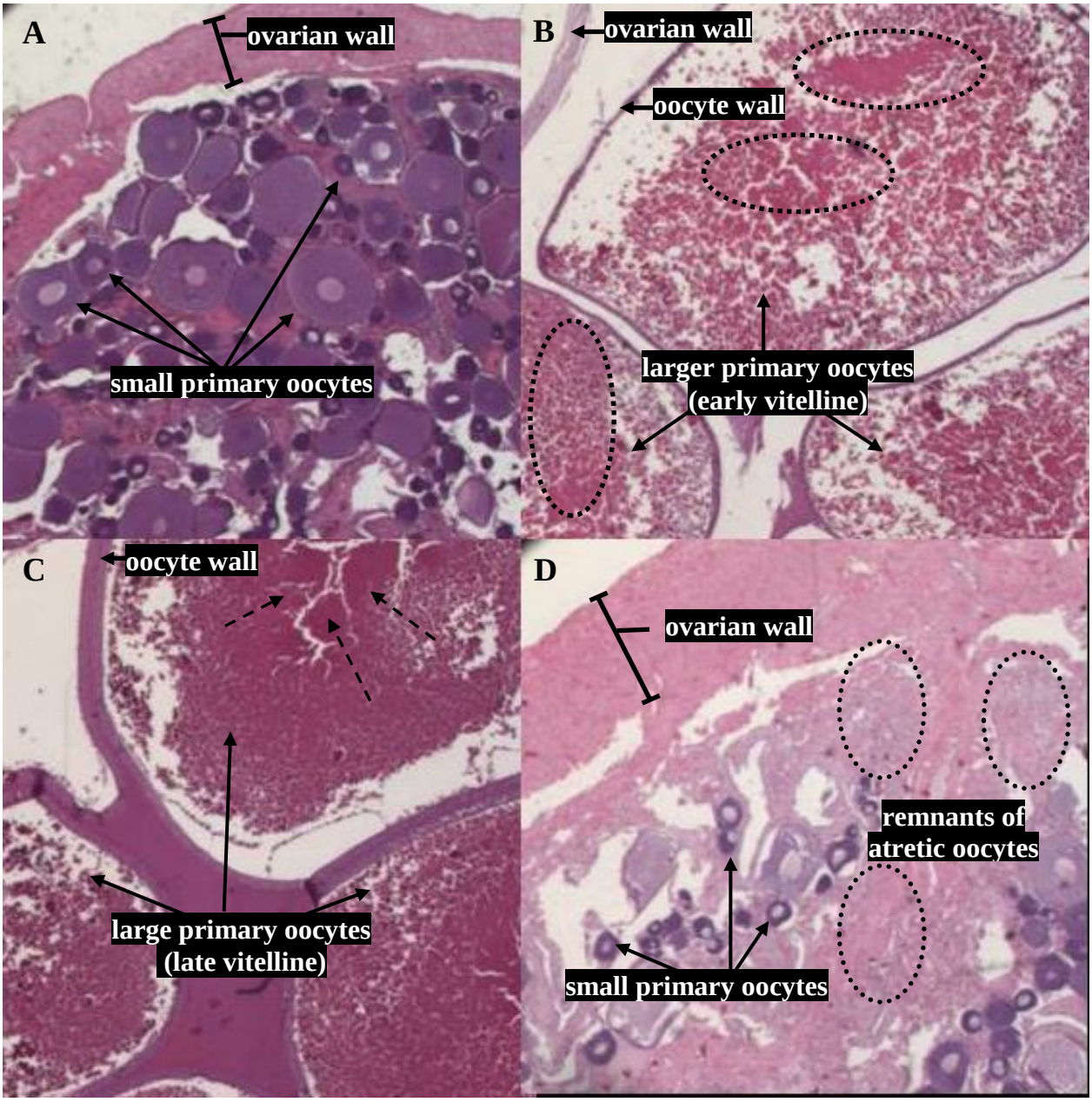


Figure C.4 Histology corresponding to ultrasound images of early (A), developing (B, C) and atretic (D) ovaries of channel catfish during the natural spawning season revealed distinct profiles. Histology corresponding to early (A) ovarian development displayed a thick ovarian wall enclosing numerous small primary oocytes with no discernible wall. Histology of developing ovaries (B, C) displayed large vitelline oocytes with a thin ovarian wall, a visible, thin oocyte wall (B), yolk globule formation (B, dotted oval shapes), a thickened oocyte wall (C), and coalescing yolk globules (C, dotted arrows). Histology of atretic (D) ovaries showed remnants of atretic oocytes, and few small primary oocytes.

development which was not visible in the ultrasound images. Ultrasound images of early and atretic ovaries (Figure C.3A, D) depicted small ovaries with no visible oocytes (Figure C.3A), or small ovaries with disfigured oocytes (Figure C.3D). In contrast, the histology profiles of early and atretic ovaries revealed a large ovarian cross-sectional area, with numerous oocytes (Figure C.4A) and distinct primary and atretic oocytes (Figure C.4D) enclosed in a thick ovarian ovarian cross-sectional area depicted in the histology profiles was directly related to the sampling of small ovaries, corresponding to early-developing or regressing ovaries (Figure C.3 A, D). Ultrasound images of developing ovaries (Figure C.3A, D) depicted a larger cross-sectional area occupied by enlarging ovaries and visible, enlarging oocytes. In contrast, the histology profiles of developing ovaries (Figure C.4 B, C), prominently displayed a large oocyte cross-sectional area, rather than a large ovarian cross-sectional area. Consequently, internal oocyte processes such as the formation and coalescence of yolk globules (Figure C.4 B, C) were depicted.

Application of ultrasound technology provides a direct, non-invasive, visualization method that can be used for evaluation of the reproductive condition of channel catfish females during the spawning season. Histological profiles corresponding to ultrasound images revealed microscopic processes that were not visible with ultrasonography, but which corroborate ultrasound imaging of ovarian development, demonstrating a strong potential utility of ultrasonography in channel catfish reproduction. Linking the ultrasound images with histology of gonadal development provides a comprehensive view of ultrasound images representative of different gonadal stages ranging from developing and developed, to advanced and atretic ovaries.

The ability to use ultrasound technology and corroborate its application with histology and other biometric indices is important in understanding the biological development of the channel catfish ovary. Identification of females in late vitellogenesis is critical for efficient hormonal induction of spawning in the hatchery. This is especially important in the application of cryopreservation to the production of hybrid catfish (channel catfish females x blue catfish *Ictalurus furcatus* males) at a commercial scale. To adequately assess and improve cryopreservation of aquatic species, technologies for understanding gonadal biology need to be incorporated into selection of females with the highest chance of ovulating fertilizable oocytes. Further studies are needed for addressing qualitative and quantitative analysis of fish gonadal development based on ultrasonography and histology to fully explore the potential application of ultrasound as a standard, non-invasive, informative means of commercial-scale assessment of a variety of fish species.

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Vita

Noel Dario Novelo, son of Adelaida Leocadia Novelo and Rodolfo Estevan Novelo, was born on October 20, 1973 in Orange Walk Town, Belize. He attended La Inmaculada Primary School in Orange Walk Town, after which he attended St. John's College (SJC) in Belize City. He obtained a high school diploma and an Associate's degree in English Literature and History from SJC. He enjoyed teaching English and Spanish at the Corozal Community College in Corozal, Belize for 5 years. He graduated with a Bachelor of Arts degree in English Literature with a minor in Natural Resource Management in 1999 at the University of Belize (UB), Belize City. There he was elected President of the UB Student Government Association, and organized rallies, parties and protests to address student issues. He was honored to receive the UB Student of the Year Award for academic performance and community service in 1999. One month after graduating from UB he obtained a research assistantship and left Belize to study public administration at Kentucky State University (KSU), Frankfort, Kentucky under the supervision of Dr. Don Anthony Woods. There he taught English as a Second Language to non-English speakers from the Caribbean and Central America, and he graduated with a Master's degree in Public Administration with a specialty in Human Resources and International Development in 2001.

Seeking to advance his multi-disciplinary training and experience, he obtained a research assistantship to study aquaculture at KSU and graduated with a Master of Science in Aquaculture/Aquatic Sciences with a specialty in Genetics and Reproduction under the supervision of Dr. Boris Gomelsky in 2008. His master's thesis was on the inheritance of DNA markers and color variability in ornamental (koi) carp *Cyprinus carpio*. During this period, he

worked at the KSU Aquaculture Research Center (Frankfort, Kentucky), and at the Thoroughbred Shrimp Hatchery (Frankfort, Kentucky). Thus he was exposed to the potential of aquaculture production to responsibly meet the food demands of the world. While at KSU, he received the Academic and Professional Achievement Award in the Public Administration program (2001), and the Outstanding Graduate Student Award in Aquaculture (2005). In pursuit of further training in aquaculture and science, he received a research assistantship in 2007 to enroll in the Wildlife and Fisheries Science doctoral program in the School of Renewable Natural Resources at Louisiana State University (LSU) in Baton Rouge, Louisiana. While at LSU, he was awarded the Ben and Pauline Stanley Excellence Award for Outstanding Graduate Students from the Department of Renewable Natural Resources (2012), the United States Aquaculture Society Presentation Award Runner-Up for oral presentation (2012), and a travel award for the Farm Animal Integrated Research conference (2012). He is grateful to all those who helped him along the way. He continues to believe in world peace, humanity, love, and in fighting against injustice and protecting our natural resources for the good of all.