# EVALUATION OF MEASUREMENT ENDPOINTS FOR THE ASSESSMENT OF POTENTIAL PETROLEUM RELATED RESPONSES BY PACIFIC HERRING: EARLY DEVELOPMENT

FINAL DRAFT REPORT

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# EVALUATION OF MEASUREMENT ENDPOINTS FOR THE ASSESSMENT OF POTENTIAL PETROLEUM RELATED RESPONSES BY PACIFIC HERRING: EARLY DEVELOPMENT

*This report is a draft of the information and conclusions made to date.*

## Introduction

On 7 November 2007 the container ship *Cosco Busan* struck a tower supporting San Francisco's Bay Bridge spilling an estimated 54,000 gallons of bunker fuel into the bay. The spill contaminated shorelines in the North Central Bay where Pacific herring would spawn in upcoming months. Historical spills in areas of Pacific herring spawning have demonstrated concordance with injury to the developing eggs and newly hatched individuals from shallow subtidal and intertidal habitats. Based on historical precedence, the importance of herring to the ecology of the bay, and their relative sensitivity to petroleum contamination, this species was selected for further examination to evaluate potential damage to natural resources in the bay. The plan was to 1) study and compare biological responses of herring embryos and larvae incubated in areas receiving different degrees of oiling from the accident and 2) to characterize the degree of exposure to polynuclear aromatic hydrocarbons (PAH), potentially resulting from the *Cosco Busan*.

Two approaches were used to assess potential damage to herring embryos from oil exposure. The first was to sample developing eggs/embryos naturally fertilized and deposited on marine vegetation along shorelines with exposured to varying degrees of the spilled bunker fuel as well as from a control site. The second was to use laboratory-fertilized eggs deployed in cages placed in shallow subtidal areas in four locations with varying degrees of exposure to the spilled bunker fuel and two reference sites. Embryos were scored for various morphological measurements and tissues were analyzed in test organisms as well as in Polyethylene Membrane Devices (PEMD) placed with the cages. Two organizations evaluated newly hatched embryo's (NOAA Fisheries and the Bodega Marine Laboratory). Data collection involved the creation of still images and video clips of live specimens. NewFields NW was asked to review the digital information and provide similar evaluations as those performed by either NOAA or BML. NewFields personnel were not informed about the locations of the samples being reviewed. Data summaries and a draft letter report were prepared by NewFields and submitted to Dr. Walter Pearson and Polaris Applied Sciences for review. After reviewing the draft letter report, NewFields was then asked to compare these data summaries to selected locations within San Francisco Bay and to evaluate whether the observations correlated with generalized descriptions of beach oiling provided by NOAA. Specific questions that were formulated as part of the review process included:

1. What is the inherent variability in the pericardial edema measurements?
2. Is more than one developmental stage represented for the artificial and/or naturally spawned embryos? If so, what are the developmental stages and how many are represented?
3. Does the attribute of yolk sac flattened (anterior edge next to pericardial space) appear in the artificial and/or naturally spawned samples? If so, what is the incidence of this attribute?
4. Are notochord abnormalities associated with the coiling or bent body axes observed in the naturally spawned samples? If so, what is the incidence of this attribute?

5. Are abnormalities other than the pericardial edema observed in the artificial spawned samples?
6. Is opacity of tissues in the natural and artificially spawned embryos an artifact of varying illumination? Is there a correlation between the background illumination level and opacity of tissues of the embryos?
7. What is the frequency of arrhythmia in artificial and naturally spawned embryos? What types of arrhythmia are present?
8. Does the frequency of abnormalities in the artificially spawned samples match those from the naturally spawned embryos?

This report describes procedures used by NewFields along with summaries of the data reviewed on a subset of the information for naturally spawned and artificially spawned samples, and an evaluation of statistical significance among and between samples collected at different sites within the bay. The summary also addresses the differences in these characteristics in association with the qualitative description of oiling along shorelines. A discussion is then provided that addresses the concordance of oiling and experimental artifacts that may be associated with the observed effects.

Location: Data were evaluated from video clips and still images of individual specimens collected from artificial or natural spawning from four locations in San Francisco Bay, California. The station designations and general locations are provided in Figure 1and Table 1.

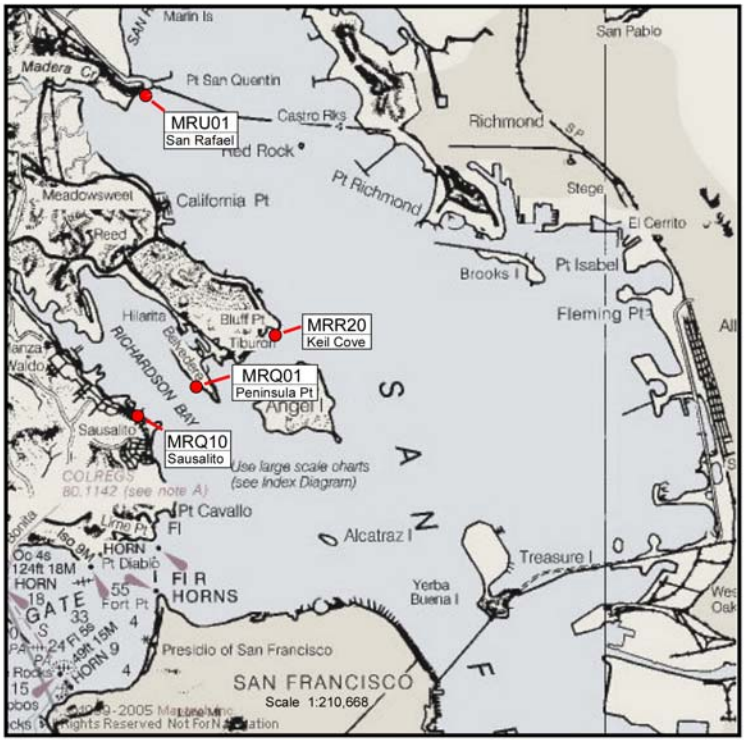



**FIGURE 1. Location of Sites Evaluated**

**TABLE 1. Station Designations, Location and Ranked Level of Intertidal and Shallow Subtidal Oiling** *(NOAA and Bodega Marine Laboratory, University of California-Davis, 2008).*

| Station Designation | Location | Rank Order of Oiling |
|---|---|---|
| MRU01 | San Rafael Bay | 0 |
| MRQ10/P01 | Sausalito | 1 |
| MRQ01 | Peninsula Point | 2 |
| MRR20 | Keil Cove | 3 |

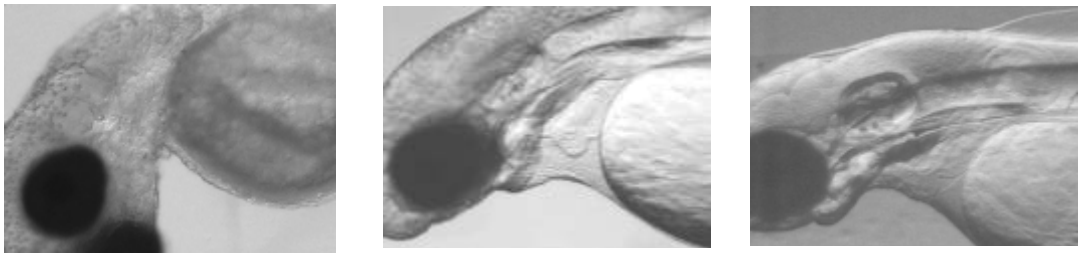
## Data Review Methods

### Morphological Characteristics

Late stage embryogenesis characteristics defined by Hill and Johnson (1997) provided the basis for assessing developmental stage through evaluation of specific characteristics such as degree of eye pigmentation, jaw and otic vesicle development, yolk sac features, pericardium shape and location, tail development, and caudal fin characteristics. Still images were previewed for selected files representing natural and artificial spawn conditions to assess gross morphological features such as planar view, degree of coiling, and relative development of tail region while video clips provided for a more detailed evaluation of head, pericardium and yolk sac regions. A description of the otic vesicle, eye pigmentation, notochord differentiation, pericardial space, heart rate and shape, heart patterns (arrhythmia), yolk sac shape and color were summarized for selected video samples and are included in Appendix A.

### Assessment of Variability

*Developmental Stage.* A subset of video files from each station location representing both natural and artificial spawned and dechorionated embryos was evaluated. Unfortunately, lack of still and video image linkage precludes a full evaluation of developmental characteristics. However, an overview of the selected embryos indicates a range of developmental growth transitioning between the organogenesis stages p, q, and r as described by Hill and Johnson. Figure 2 presents examples of the developmental stages (p-q) encountered in our review; Table 2 summarizes distinctive characteristics.



A. Early Stage [p, MRQ01 N6 Clip 06] B. Intermediate [q, MRQ01 A1 Clip 05] C. Pre-Hatch Stage [q, MRU01 N2 Clip

**FIGURE 2. Range of Developmental Characteristics**

**Table 2. Notable Characteristics of Pre-Hatch Developmental Stages** *(based on Hill and Johnston 1997)*

| Diagnostic Characteristic | Early (p-Stage) | Intermediate to Pre-Hatch (q-Stage) |
|---|---|---|
| Days Post Fertilization (dpf) | 7 - 10 dpf | 11- 14 dpf |
| Length | 3- 5 mm | 5-7 mm |
| Eye Pigmentation | 10% - 75% | 85 – 100% |
| Head/Trunk Angle (HTA) | 50° | 130° - 145° |
| Hatching glands | Cover dorsal regions of head | Hatching glands release contents |
| Otic Vesicle | Otoliths developed; primordia of semi-circular canals develop | Semi-circular canals in otic vesicles are more prominent |
| Pectoral Fin | Pectoral fin bud develops | Pectoral fin develops into functional structure |
| Jaw Development | Rudimentary | Protrusion evident, may fully extend under eye |
| Pericardium Shape, and Orientation | Elongated; anterior portion of pericardial space | Atrium follows vertical axis and ventricle follows horizontal axis, oriented in center of pericardial region |
| Yolk sac shape | Rounded | Elliptical |
| Notochord development | Rudimentary – segmentation not clearly defined | Segmentation notable |

*Specimen Orientation.* Many still images from the natural spawn groups demonstrated various degrees of coiling, generally considered a sign of abnormal development. We noted basic shape differences as shown in Figure 3. However, it should be clarified whether presence of early developmental stage at time of dechorionation may contribute to some degree of residual coiling, and/or whether salinity stress (i.e., ambient salinity at time of collection versus laboratory salinity) may have impacted progression of developmental stages.

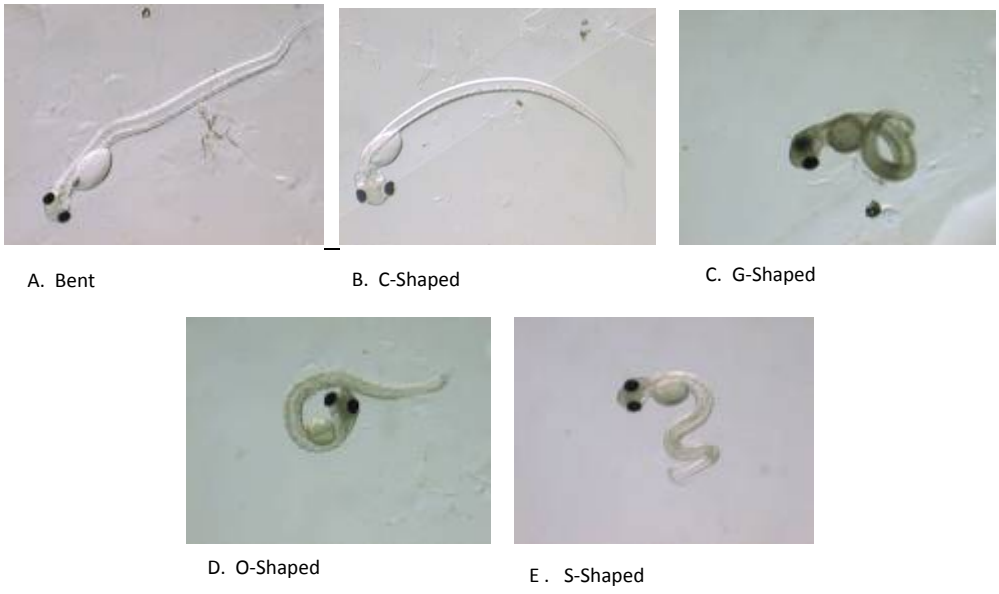



**Figure 3. Various Specimen Orientations**

Some instances of anomalous notochord development were observed (Figure 4); however these images were not directly associated with corresponding video recordings, and thus a full assessment of abnormality could not be made. Based on the description of abnormality of notochord associated with a 90° bend in the notochord beyond the dorsal, ventral, or lateral axis (Hershberger et al. 2005), or a constriction of the notochord, the specimens presented in Figure 4 are probably anomalous. We reviewed the still images from each site and summarized the frequency of body axis deviations from the horizontal plane, commonly encountered in review of naturally spawned larvae. This is a qualitative assessment, and caution should be exercised when interpreting these data. A quantitative evaluation of frequency of anomalies occurring to the notochord is very restricted due to the lack of appropriate resolution of the full specimen still images.

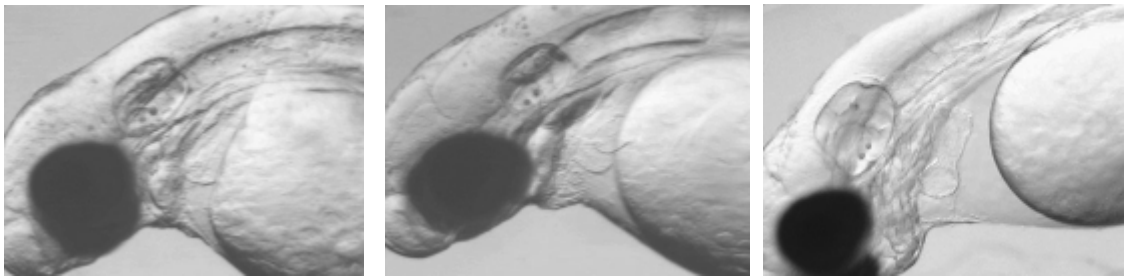
**Figure 4. Anomalous Notochord Development**

*Illumination.* An assessment of the variability of illumination was made on a select subset of video clips. Points were measured from the background, the head region (including the otic vesicle), the eye, and the yolk sac using ImageJ software to analyze median gray values. The degree of illumination and pigmentation in these regions of the individual specimens were compared using regression analysis.

A cursory scan of the full image collection indicated that the still and video recordings were made under various lighting conditions. This variation in illumination makes an evaluation of relative opacity of the various areas of the specimen problematical. Variation in illumination encountered during review of the still images is exemplified in Figure 5. Very frequently, a gold-tone cast to specimen captured in still photos was noted, particularly if there was some degree of coiling in body orientation (e.g., photo at far right below).

**Figure 5. Variation in Background Illumination in Still Images**

*Position of the Heart Relative to Yolk Sac* A large variation in the size of the pericardium and position of the heart relative to the yolk sac was observed in the subset of video recordings included in this review (Figure 6; flattening of the yolk sac may or may not be evident).



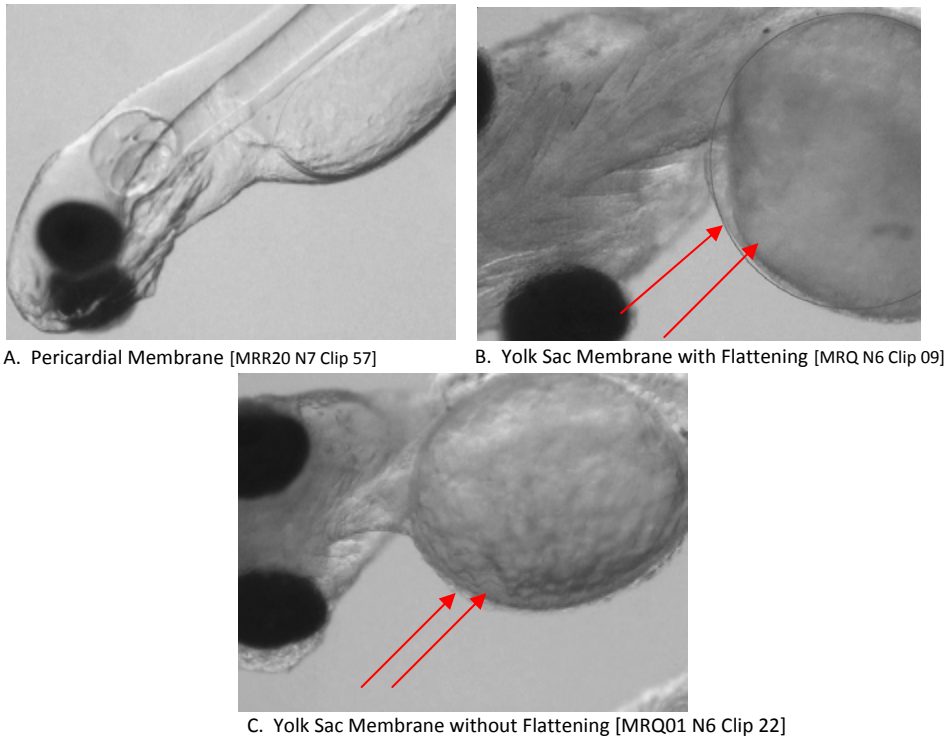
A. Compressed Pericardium with Flattening [MRQ10 A3 Clip 32]

B. Overlap of Heart onto Yolk Sac without Flattening [MRQ10 A5 Clip 20]

C. Large Pericardium with Anterior Orientation of Heart [MRR20 N4 Clip 09]

**FIGURE 6. Variations in Pericardial Region**

In addition, instances of secondary membranes were noted in the pericardial region as well as the yolk sac as shown in Figure 7. The determination of flattened yolk sac was based on a deviation from the round or elliptical shape. In cases where flattening was not easily observed, Image J software was used to make a rounded or elliptical tool to follow the outline of the yolk sac. There are two associations of flattening with an extra space around the yolk sac. In the first instance, flattening occurs in an absence of an extra clear space around the yolk sac, and in the later case it occurs in the presence of a extra space around the yolk sac "edema". Figure 7B illustrates yolk sac flattening associated with an extra space around yolk sac. The observations are recorded in Appendix A5.



A. Pericardial Membrane [MRR20 N7 Clip 57]

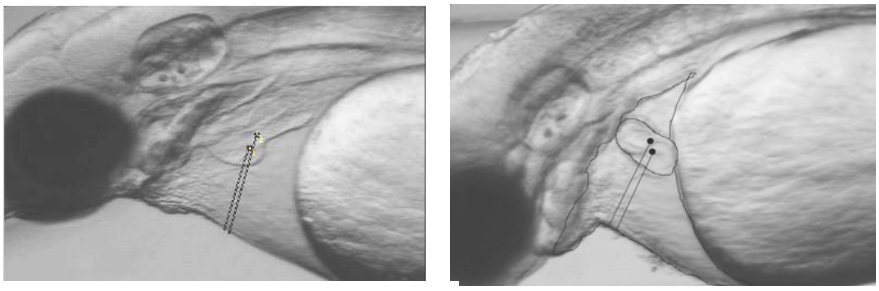
B. Yolk Sac Membrane with Flattening [MRQ N6 Clip 09]

C. Yolk Sac Membrane without Flattening [MRQ01 N6 Clip 22]

**FIGURE 7. Additional Observations**

The distance of the heart atrioventricular junction (AV) to the ventral edge of pericardial space was measured using a perpendicular line for subset of videos using ImageJ software. Measurements were taken on the downbeat and the upbeat of the heart and differences in measurement were assessed statistically. In addition, the area of the heart and pericardial space

were measured using ImageJ software. The data have been summarized and provided in Appendix A and are illustrated in Figure 8 A and B. The variation in distance based on position of the heartbeat was determined for selected video clips for each of the station/areas indicated.



A. AVPC distance to ventral edge of pericardium [MRQ10P01A2-Clip 3]]

B. Area of Heart and Pericardial Region [MRQ01-A1-10-Clip 04]

**Figure 8. Measurement of Distance from Upbeat/Downbeat AV to PC (A); Measurement of Area of Heart and Pericardium (B)**

*Heart Beat and Arrhythmia*. The number of heart beats for 20 second intervals was counted and recorded for each video clip evaluated. Incidence of arrhythmia was determined as the presence of an irregular beating or compression of the atrium or ventricle during a 20 second video clip.

*Heart and Pericardial Space Areas.* Computer based planimetry was employed using ImageJ to determine the area of heart, the area of pericardial space, and the percentage of pericardial space occupied by the heart.

*Statistical Evaluation Procedures*

ANOVA was performed on each of the quantified data sets. Linear regression was performed on the associations between background illumination and density of pigmentation in tissues as well as regressions of the measurements to the rank order of qualitative oiling.

## Results

Morphological Characteristics: Still Image Documentation *(Low Resolution)*

The full set of still images for naturally spawned embryos was assessed for body axis deviations. We recorded the various degrees of coiling as C-, O-, or S-shaped; non-coiled specimen were considered linear; and those with >90° bends along the notochord or other anomalous aspects to notochord development were grouped as "body axis defect". These results are summarized below in Table 3, and illustrated in Figure 9A. Figure 9B shows results of the NewFields assessment compared to results reported from a similar analysis conducted by NOAA (NOAA/BML 2008). It should be noted that all non-straight embryos were classified as having body axis defect for the NOAA/BML study, whereas the NewFields assessment included only those specimens with verifiable notochord irregularities.

**Table 3. Assessment of Body Axis Irregularities**

| Site | Body Axis Defect | C-Shaped | G-Shaped | O-Shaped | S-Shaped | Linear | NM * | Σ Non Linear/ Total # | Total # Assessed |
|---|---|---|---|---|---|---|---|---|---|
| MRQ01--N1- N8 | 84 | 2 | 4 | 20 | 23 | 2 | 26 | | **135** |
| Mean | 52% | | | | | | | 99% | |
| STD | 6% | | | | | | | 2% | |
| CV% | 12% | | | | | | | 2% | |
| MRQ10- N1 - N8 | 107 | 5 | 2 | 5 | 2 | 36 | 9 | | **157** |
| Mean | 64% | | | | | | | 78% | |
| STD | 22% | | | | | | | 23% | |
| CV% | 34% | | | | | | | 29% | |
| MRR20- N1 -N8 | 122 | 1 | 2 | 15 | 5 | 11 | 7 | | **156** |
| Mean | 75% | | | | | | | 94% | |
| STD | 17% | | | | | | | 10% | |
| CV% | 22% | | | | | | | 11% | |
| MRU01-N1 -N8 | 15 | 5 | 0 | 0 | 0 | 138 | 0 | | **158** |
| Mean | 9% | | | | | | | 12% | |
| STD | 13% | | | | | | | 16% | |
| CV% | 142% | | | | | | | 129% | |

**NM = not measured * % non-linear is equivalent to the determinations made by NOAA*

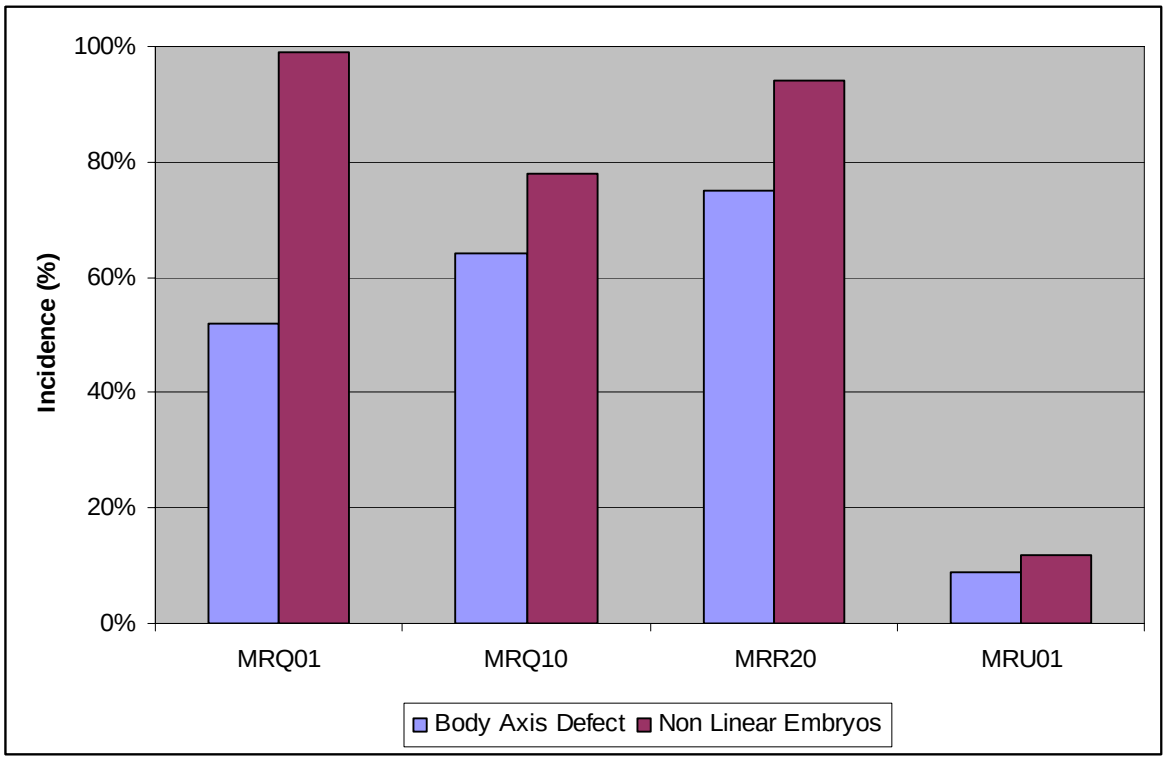



**Figure 9A. Assessment of Body Axis Irregularities.**
*(blue bars represent % with distinct body defects; maroon bars represent total % non-linear embryos)*

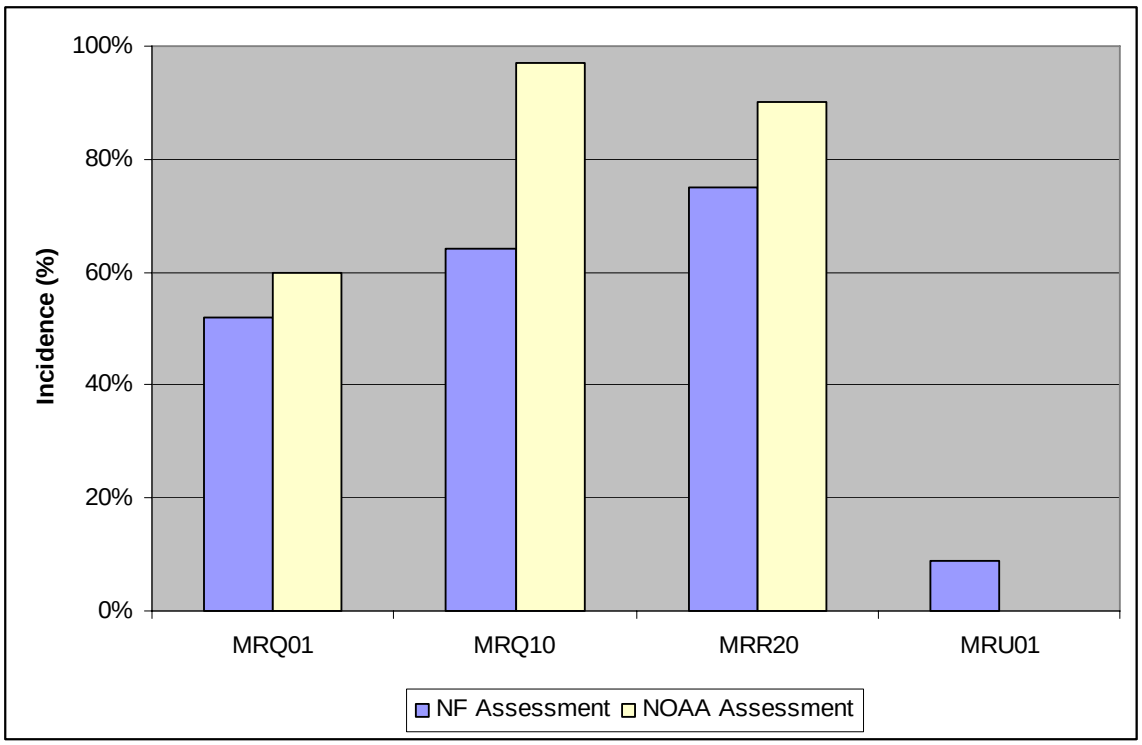



**Figure 9B. Comparison of Incidence of Notochord Irregularities**
*[blue bar presents data from this study; yellow bars represent data records presented by NOAA (NOAA and BML, 2008)]*

Morphological Characteristics: Video Documentation

Results of the descriptive statistics (range, mean, standard deviation and coefficient of variation) and the statistical differences within and between stations were recorded for a subset of available videos representing each site location. The following parameters were evaluated and are summarized in Table 4.

1. Qualitative description of larval stages in natural and artificial spawned specimens
2. Measurement of the length and difference in length (pixels) of upbeat and downbeat AV to PC (reported as Potential % Difference) calculated by comparing the maximum length – the minimum length divided by the mean length.
3. Heart Rate per 20 seconds
4. Heart Area (pixels)
5. Pericardial Region (pixels)
6. Heart to Pericardial Region Ratio
7. Yolk Flattening (% Incidence)
8. Arrhythmia (% Incidence)

Table 5 contains the results of the ANOVA designating statistically significant differences in mean measurements for the stations; Figures 10-15 provide visual displays of the data.

**Table 4. Summary of Statistical Evaluation of Dechorionated Embryo Metrics**

| Range | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Measurement | MRU01 | | MRQ10/P01 | | MRQ01 | | MRR20 | |
| | Natural | Artificial | Natural | Artificial | Natural | Artificial | Natural | Artificial |
| AVPC Length (min and max) | 36 to 81 | 35 to 92 | 45 to 94 | 80 to 165 | NM | 34 to 148 | 49 to 100 | 51 to 116 |
| AVPC Potential % Difference | 7 TO 18 | 7 to 24 | 6 TO 27 | 17 to 34 | NM | 13 to 21 | 9 TO 20 | 0 to 23 |
| Heart Rate per 20 seconds | 34 TO 42 | 31 to 42 | 34 TO 48 | 28 to 36 | 11 TO 31 | 28 to 34 | 34 TO 50 | 25 to 45 |
| Heart Area (pixels) | 8063 TO 12744 | 4914 to 10898 | 6464 TO 10713 | 6603 to 11108 | NM | 5973 to 10752 | 8194 TO 8913 | 5606 to 11145 |
| Pericardial Region (pixels) | 19242 TO 42087 | 14576 to 31419 | 13612 TO 31904 | 21986 to 54305 | NM | 15977 to 35196 | 26509 TO 35472 | 10652 to 30132 |
| Heart to Pericardial Region (%) | 26 TO 61 | 33 to 46 | 29 TO 60 | 18 to 39 | | 17 to 51 | 24 TO 34 | 29 to 73 |
| Yolk Sac Flattening (Incidence %) | Not applicable | | | | | | | |
| Arrhythmia (Incidence %) | Not applicable | | | | | | | |
| Larval Stage | q | q | p and q | p and q | p | q | p and q | p and q |
| Oiling | No oiling | | Very light to light | | Light | | Heavy to light | |
| Location | San Rafael | | Sausalito | | Peninsula Pt | | Keil Cove | |
| Oiling Rank | 0 | | 1 | | 2 | | 3 | |
| **Mean** | | | | | | | | |
| | MRU01 | | MRQ10/P01 | | MRQ01 | | MRR20 | |
| Measurement | Natural | Artificial | Natural | Artificial | Natural | Artificial | Natural | Artificial |
| AVPC Length (min and max) | 56 | 60 | 62 | 111 | NM | 72 | 70 | 86 |
| AVPC Potential % Difference | 28 | 29 | 26 | 19 | NM | 28 | 22 | 19 |
| Heart Rate per 20 seconds | 37 | 36 | 39 | 31 | 22 | 31 | 42 | 36 |
| Heart Area (pixels) | 11269 | 8885 | 8642 | 9040 | NM | 8234 | 8518 | 8521 |
| Pericardial Region (pixels) | 31325 | 23781 | 22677 | 35205 | NM | 24205 | 30665 | 20930 |
| Heart to Pericardial Region (%) | 38 | 38 | 40 | 27 | NM | 37 | 28 | 44 |
| Yolk Sac Flattening (Incidence %) | 37 | 28 | 38 | 31 | NM | 18 | 56 | 27 |
| Arrhythmia (Incidence %) | 11 | 0 | 11 | 0 | 0 | 17 | 33 | 33 |
| Larval Stage | 100 q | 100q | 33 p; 67 q | 100 q | 100 p | 100 q | 67 p; 33q | 56 p; 44 q |
| Oiling | No oiling | | Very light to light | | Light | | Heavy to light | |
| Location | San Rafael | | Sausalito | | Peninsula Pt | | Keil Cove | |
| Oiling Rank | 0 | | 1 | | 2 | | 3 | |
| NM = Not Measurable | | | | | | | | |

**Table 4. Summary of Statistical Evaluation of Dechorionated Embryo Metrics** *(Continued)*

| STD | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Measurement** | **MRU01** | | **MRQ10/P01** | | **MRQ01** | | **MRR20** | |
| | **Natural** | **Artificial** | **Natural** | **Artificial** | **Natural** | **Artificial** | **Natural** | **Artificial** |
| AVPC Length (min and max) | 12.2 | 13.8 | 13.7 | 22.2 | NM | 35 | 21.3 | 8.4 |
| AVPC Potential % Difference | 6 | 10 | 12 | 5 | NM | 9 | 12 | 12 |
| Heart Rate per 20 seconds | 2 | 4 | 5 | 2 | 8 | 2 | 6 | 7 |
| Heart Area (pixels) | 1328 | 2030 | 1554 | 1189 | NM | 1606 | 364.8 | 1946 |
| Pericardial Region (pixels) | 7417 | 5544 | 5785 | 10168 | NM | 7786 | 4517 | 7262 |
| Heart to Pericardial Region % | 12.7 | 5 | 10 | 7 | NM | 12 | 5 | 13 |
| Yolk Sac Flattening (Incidence %) | 2.9 | 8 | 10 | 4 | NM | 2 | | 7 |
| Arrhythmia (Incidence %) | 19 | 0 | 19 | 0 | 0 | 23.4 | 47 | 57.7 |
| Larval Stage | Not applicable | | | | | | | |
| Oiling | No oiling | | Very light to light | | Light | | Heavy to light | |
| Location | San Rafael | | Sausalito | | Peninsula Pt | | Keil Cove | |
| Oiling Rank | 0 | | 1 | | 2 | | 3 | |
| **CV%** | | | | | | | | |
| | **MRU01** | | **MRQ10/P01** | | **MRQ01** | | **MRR20** | |
| **Measurement** | **Natural** | **Artificial** | **Natural** | **Artificial** | **Natural** | **Artificial** | **Natural** | **Artificial** |
| AVPC Length (min and max) | 22 | 23 | 22 | 20 | NM | 49 | 30 | 22 |
| AVPC Potential % Difference | 23 | 34 | 45 | 25 | NM | 33 | 56 | 62 |
| Heart Rate per 20 seconds | 5.4 | 10 | 12 | 8 | 38 | 7 | 15 | 18 |
| Heart Area (pixels) | 12 | 23 | 18 | 13 | NM | 20 | 4 | 23 |
| Pericardial Region (pixels) | 24 | 23 | 26 | 29 | NM | 32 | 15 | 45 |
| Heart to Pericardial Region % | 33 | 14 | 25 | 25 | NM | 32 | 18 | 4 |
| Yolk Sac Flattening (Incidence %) | 7.9 | 30 | 35 | 12 | NM | 13 | 0 | 25 |
| Arrhythmia (Incidence %) | 173 | 0 | 173 | 0 | 0 | 141 | 141 | 173 |
| Larval Stage | Not applicable | | | | | | | |
| Oiling | No oiling | | Very light to light | | Light | | Heavy to light | |
| Location | San Rafael | | Sausalito | | Peninsula Pt | | Keil Cove | |
| Oiling Rank | 0 | | 1 | | 2 | | 3 | |

**Table 5. Results from ANOVA Analyses** *(Stations underlined with the same color are not significantly different)*.

| Measurement | Prob > F | Mean Measurement (Highest to Lowest) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| AVPC Length (min and max) | <0.001 | Artificial MRQ10/P01 | Artificial MRR20 | Artificial MRQ01 | Natural MRR20 | Natural MRQ10/P01 | Artificial MRU01 | Natural MRU01 | Natural MRQ01 |
| | | 111 | 86 | 72 | 70 | 62 | 60 | 56 | No Data |
| Heart Beat Rate | <0.001 | Natural MRR20 | Natural MRQ10/P01 | Natural MRU01 | Artificial MRR20 | Artificial MRU01 | Artificial MRQ01 | Artificial MRQ10/P01 | Natural MRQ01 |
| | | 42 | 39 | 37 | 36 | 36 | 31 | 31 | 22 |
| Heart Area (HA) | 0.007 | Natural MRU01 | Artificial MRQ10/P01 | Artificial MRU01 | Natural MRQ10/P01 | Artificial MRR20 | Natural MRR20 | Artificial MRQ01 | Natural MRQ01 |
| | | 11269 | 9040 | 8885 | 8642 | 8521 | 8518 | 8234 | No Data |
| Pericardial Space (PS) | 0.001 | Artificial MRQ10/P01 | Natural MRU01 | Natural MRR20 | Artificial MRQ01 | Artificial MRU01 | Natural MRQ10/P01 | Artificial MRR20 | Natural MRQ01 |
| | | 35205 | 31325 | 30665 | 24205 | 23781 | 22677 | 20930 | No Data |
| HA/PS | 0.022 | Artificial MRR20 | Natural MRQ10/P01 | Natural MRU01 | Artificial MRU01 | Artificial MRQ01 | Natural MRR20 | Artificial MRQ10/P01 | Natural MRQ01 |
| | | 44% | 40% | 38% | 38% | 37% | 28% | 27% | No Data |
| Yolk Flattening (%) | 0.007 | Natural MRR20 | Natural MRQ10/P01 | Natural MRU01 | Artificial MRQ10/P01 | Artificial MRU01 | Artificial MRR20 | Artificial MRQ01 | Natural MRQ01 |
| | | 56% | 38% | 37% | 31% | 28% | 27% | 18% | No Data |

*Qualitative Description of Larval Stages in Natural and Artificial Spawnings.* The degree of embryo development in natural and artificially spawned samples generally represents transitional characteristics of stages *p* through pre-hatch (*q*). The artificially spawned embryos represent the intermediate stage (*q*; Figure 2b, Table 4) while the naturally spawned embryos represent more variation, ranging from younger (*p*) to older stages (*q*) (Figure 2a-c). Data from the natural spawned specimens from station MRQ01 represent earlier stages of development for all of the samples that were reviewed while the remaining stations with naturally spawned specimens represent more fully developed embryos (Table 4). Dechorionated embryos from naturally spawned eggs collected from station MRQ01 were the most problematic for scoring; most morphological characteristics were not visible for this group. Qualitatively, there is a difference between the larvae evaluated for the artificially and the naturally spawned specimens.

*Potential Differences in AVPC Length.* The video images provide a method of measuring the difference in AVPC length based on upbeat or downbeat of the heart within the pericardial space. This variation was calculated for each specimen examined using individual video clip images; thus providing an assessment of the natural variation associated with a given specimen based on the beat of the heart at the time of the measurement. The variation in the AVPC length for the

four study areas based on position of heart is 25% for natural and 36% for artificially spawned samples. The length of the beat distance is longer for the artificially spawned embryos compared to the naturally spawned embryos. This difference is associated with the observations from the artificially spawned larvae examined at station MRQ10/P01 (Appendix A). Results of ANOVA indicate that this station is statistically different from all others; MRR20 is statistically different from naturally spawned embryos from MRQ10 and MRU01 as well as the artificial spawned embryos from MRU01 as shown in Table 5. Anecdotal observations of the larval stage at MRQ10 indicate the artificially spawned embryos are probably at a later stage than most of the naturally spawned samples and consequently, the significant difference noted may be associated with the relative stage of development of the embryos.

The differences between the lengths of an up- and downbeat are not statistically significantly different between those samples that were artificially or naturally spawned, nor are there statistically significant differences among the various stations (Appendix A). A biologically important difference is observed only if the length of AVPC distance among or between stations is greater than the variation within beat lengths for individual heart beats. This was examined by comparing variation within a subset of artificially and naturally spawned samples for each station. The number of specimens with longer beat lengths than expected within a station or compared by all stations was 2 or 3 instances per station; however, the incidence of longer beat lengths was consistently higher for artificially spawned samples from MRQ10/P01. These measurements were consistently larger and appear to represent an older larval stage than other location/spawning group assessments (Figure 10).

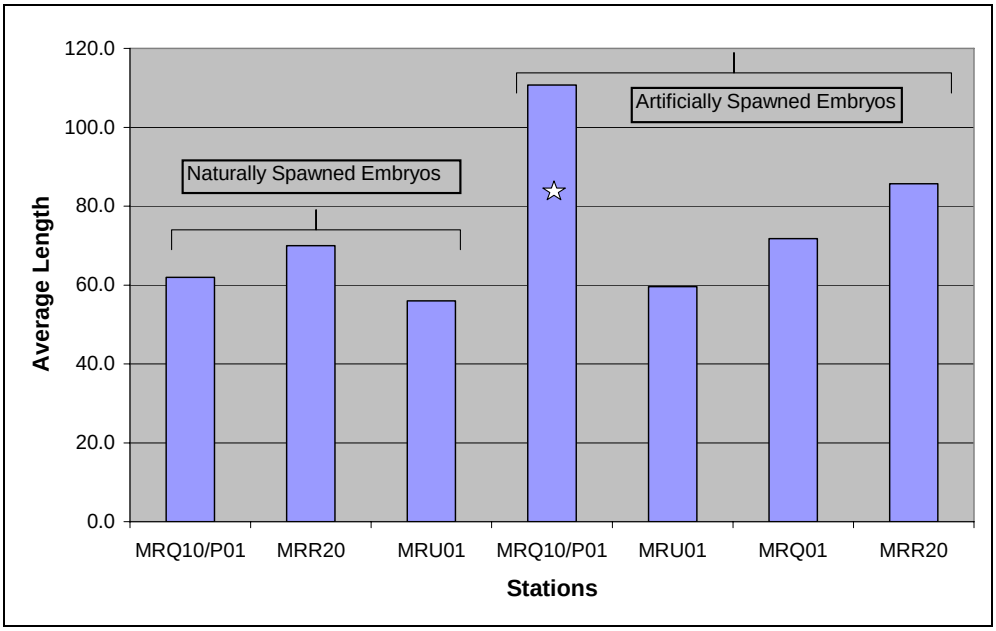



**FIGURE 10. ANOVA Results of Differences in AVPC Measurements**
*[Star indicates statistically significant from all other stations]*

*Heart Rate and Arrhythmia.* The heart rate of the samples evaluated ranged from 11 to 50 beats per 20 second video clip. The heart rate for specimens collected from the natural spawn at station MRQ01 were significantly lower than those at all other stations. The variation of heart rates for naturally spawned samples from MRQ01 is consistent with the fact that all samples evaluated were early p-larval stage. The range of heart rate values observed with both the natural and artificial spawn at station MRR20 is larger than the other stations as measured by CV%; and these

samples also represent a larger percentage of p-larval stages (56 to 67%). Figure 11 shows the average heart rate per station/spawning group.

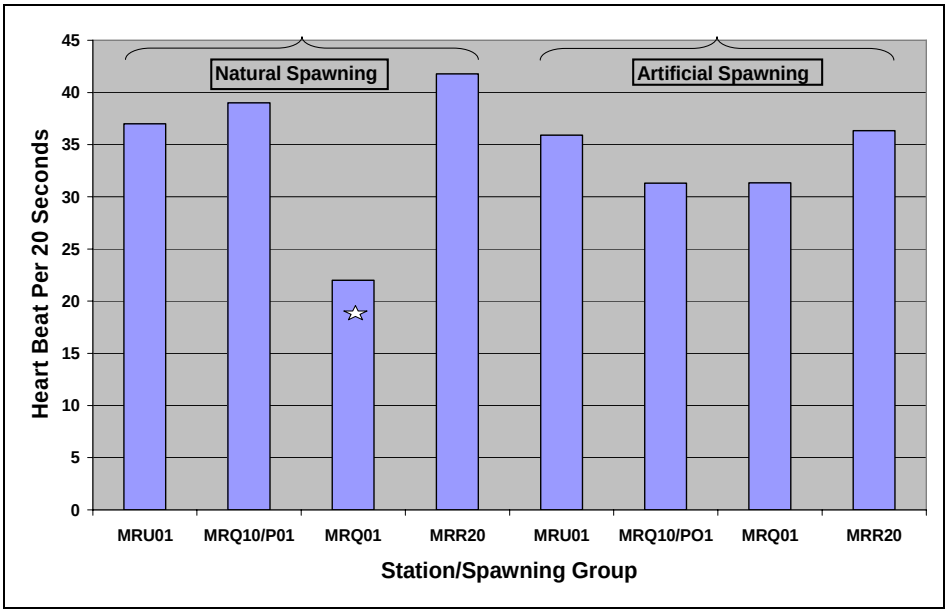



**FIGURE 11. Analysis of Heart Rate**

*[Star indicates statistically significant from all other stations]*

The incidence of heart arrhythmia for the naturally spawned and artificially spawned samples from a subset of each of four stations was 0-33%. The incidence of arrhythmia in artificially spawned embryos was 0% at MRU01 and MRQ10/P01, 17% at MRQ01, and 33% at MRR20. The incidence of arrhythmia in naturally spawned embryos was 0% at MRQ01; 11% at MRU01 and MRQ10; and 33% MRR20. Figure 12 illustrates the incidence of arrhythmia by station.

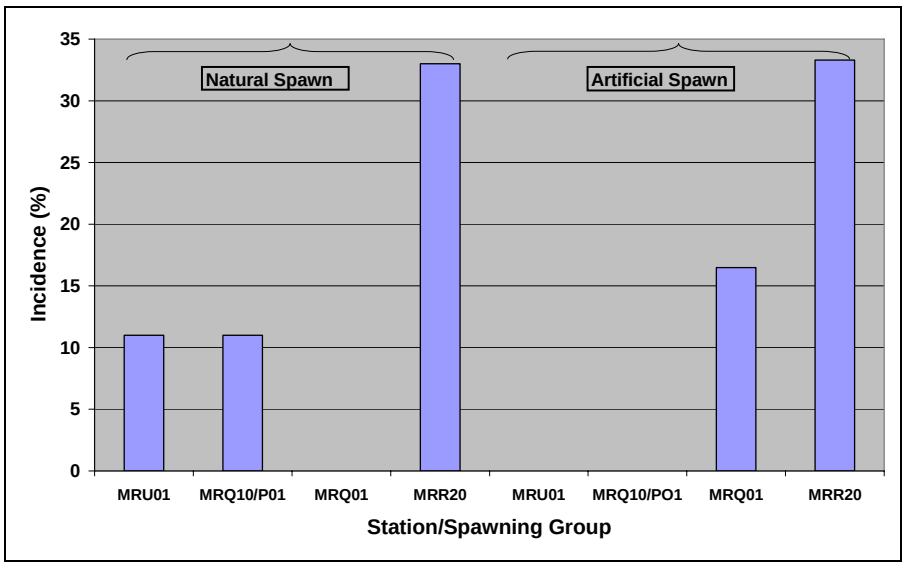



**FIGURE 12. Incidence of Arrhythmia**

*Heart Area, Pericardial Region and Heart to Pericardial Region Ratio.* As the heart increases in size the pericardial space will either increase, maintaining a similar ratio; or will increase or decrease in size relative to the size of the heart. Pericardial edema is based on a swelling of the space within this region. Figures 13 and 14 present the average size of the heart, the average size of the pericardial space and the ratio of the heart to the pericardial space. The heart area for natural spawn MRU01 was significantly larger than artificial MRR20, natural MRR20, and artificial MRQ01 (Table 5). Pericardial space was significantly larger in artificial MRQ10/P01 than in artificial MRR20.

*Yolk Sac Flattening (% Incidence).* An apparent result of increasing heart or pericardial space area is a flattening of the yolk sac on the anterior face. The incidence of yolk sac flattening is relatively the same across natural and artificial spawning groups at all stations except MRR20 where the natural spawn flattening was significantly higher flattening incidence of artificially spawned samples from MRR20 (Figure 15).

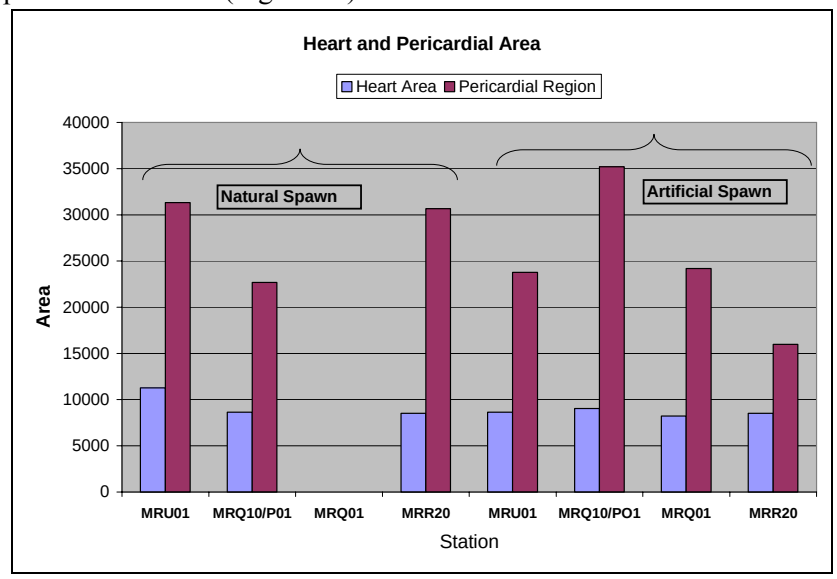



**FIGURE 13. Comparison of Heart and Pericardial Areas** *(pixels)*

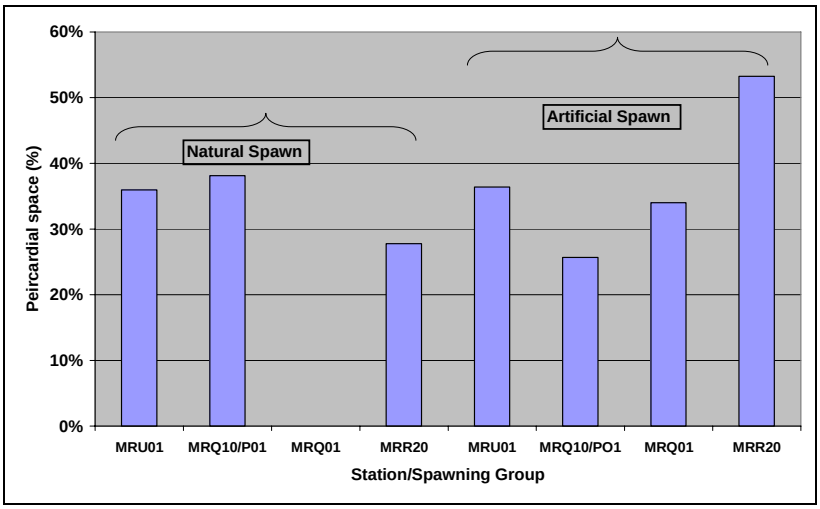



**FIGURE 14. Ratio Heart: Pericardial Space**

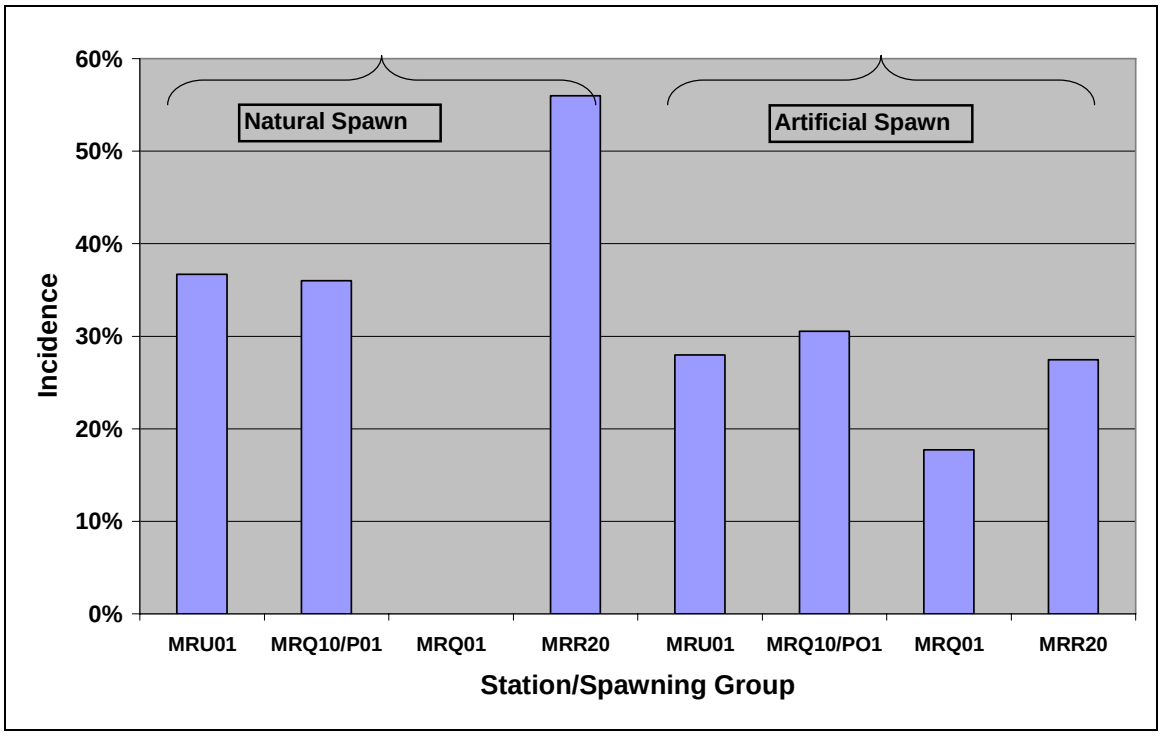



**FIGURE 15. Observed Incidence of Flattened Yolk Sacs**

*Background Illumination.* Data produced from measurement of mean gray values at several locations within individual video clips are presented in Table 6 and 7. The relationship between background illumination and illumination of various tissues (otic vesicle, eye, and yolk sac) are summarized in Figures 16 through 18. For features that are lighter (higher gray scale values) in color (otic vesicle and yolk sac) there are significant regressions ($p<0.05$) between the background illumination and the feature. There does not appear to be a difference in the relationship to background illumination for each feature between the natural and artificially spawned embryos.

**Table 6. Measured Mean Gray Values from Natural Spawn Video Recordings** *(first frame)*

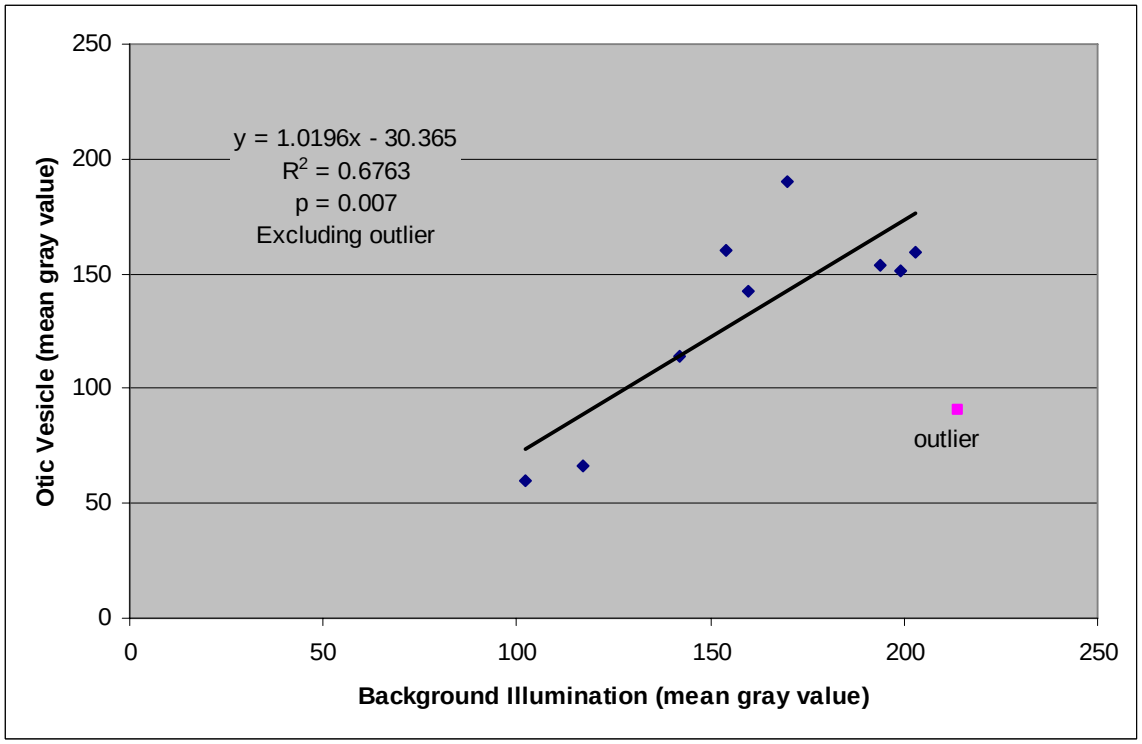
| Natural Spawn | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Site | Video Clip # | Illumination *(mean gray value*)* | | | | | | | | |
| | | Background | | | Head | | | Eye | | Yolk Sac |
| | | Top Left | Bottom Rt | Bottom Left | Dorsal | Otic Vesicle | Dorsal, 2nd | Center | Outside | Not in Shadow |
| MRQ01-FEM1-022908-N4 | 11 | 194 | 210 | 206 | 118 | 154 | 146 | 27 | 37 | 159 |
| MRQ01-FEM1-022908-N6 | 6 | 199 | 201 | 206 | 134 | 151 | 132 | 22 | 30 | 147 |
| MRQ10/P01-FEM1-022708-N2 | 91 | 142 | 154 | 155 | 162 | 114 | 114 | 33 | 39 | 183 |
| MRQ10/P01-FEM1-022708-N6 | 10 | 170 | 186 | 188 | 198 | 190 | 105 | 20 | 30 | 171 |
| MRQ10/P01-FEM1-022708-N8 | 5 | 154 | 161 | 128 | 211 | 160 | 130 | 21 | 27 | 157 |
| MRR20-FEM1-022808-N4 | 9 | 203 | 209 | 216 | 241 | 159 | 149 | 22 | 27 | 234 |
| MRR20-FEM1-022808-N7 | 51 | 214 | 217 | 210 | 135 | 91 | 129 | 34 | 47 | 205 |
| MRU01-FEM1-022608-N2 | 3 | 102 | 128 | 126 | 121 | 60 | 78 | 22 | 25 | 123 |
| MRU01-FEM1-022608-N4 | 5 | 160 | 161 | 157 | 136 | 142 | 111 | 20 | 22 | 173 |
| MRU01-FEM1-022608-N7 | 8 | 117 | 145 | 138 | 172 | 66 | 201 | 22 | 27 | 140 |
| Mean | | 165.5 | 177.2 | 173.0 | 162.8 | 128.7 | 129.5 | 24.3 | 31.1 | 169.2 |
| STD | | 37.7 | 31.3 | 36.0 | 41.9 | 43.8 | 32.7 | 5.2 | 7.6 | 32.4 |
| CV | | 23% | 18% | 21% | 26% | 34% | 25% | 22% | 25% | 19% |

**Low numbers represent dark tones; high numbers represent light tones, gray values range from 0 to 255.*

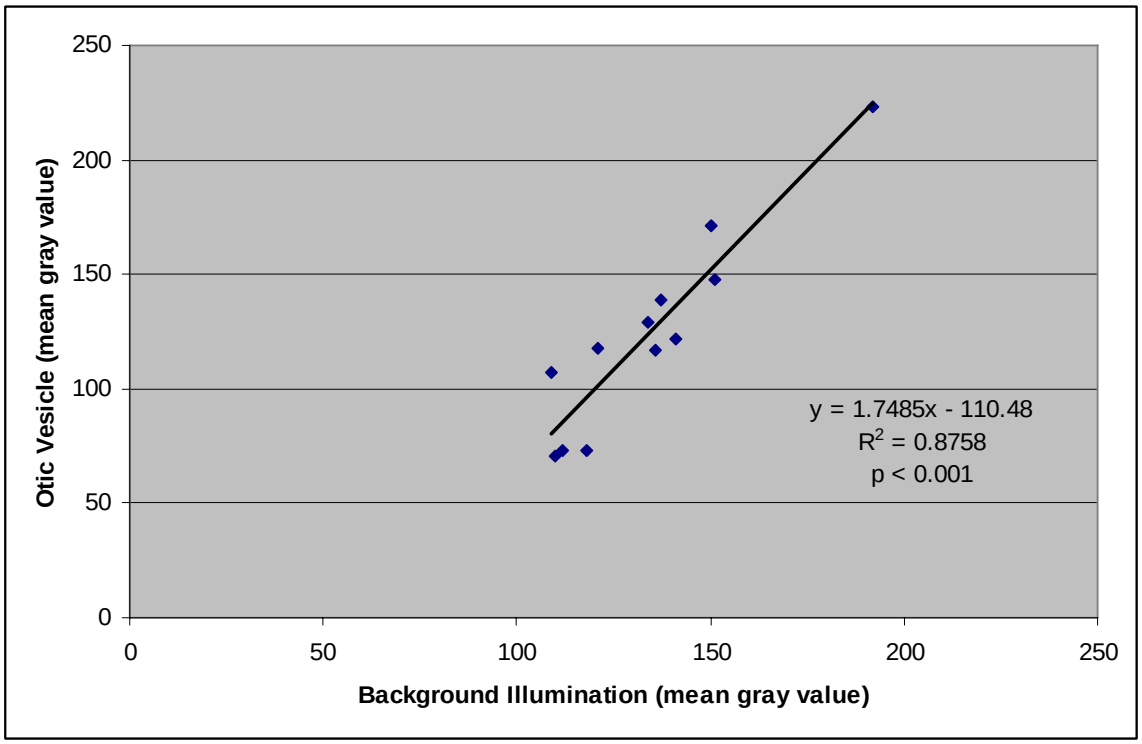
**Table 7. Measured Mean Gray Values from Artificial Spawn Video Recordings** *(first frame)*

| Artificial Spawn | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Site | Video Clip # | Illumination *(mean gray value*)* | | | | | | | | |
| | | Background | | | Head | | | Eye | | Yolk Sac |
| | | Top Left | Bottom Right | Bottom Left | Dorsal | Otic Vesicle | Dorsal, 2nd | Center | Outside | Not in Shadow |
| MRQ01-FEM1-021808-A1 | 4 | 137 | 153 | 153 | 155 | 139 | 100 | 17 | 24 | 159 |
| MRQ01-FEM1-021808-A3 | 8 | 136 | 129 | 135 | 116 | 117 | 105 | 17 | 25 | 141 |
| MRQ10/P01-FEM1-021908-A1 | 1 | 151 | 174 | 174 | 131 | 148 | 85 | 20 | 22 | 172 |
| MRQ10/P01-FEM1-021908-A2 | 3 | 110 | 140 | 137 | 149 | 71 | 107 | 17 | 29 | 139 |
| MRQ10/P01-FEM1-021908-A3 | 29 | 118 | 141 | 145 | 154 | 73 | 82 | 17 | 26 | 156 |
| MRQ10/P01-FEM1-021908-A5 | 1b | 134 | 146 | 146 | 168 | 129 | 119 | 19 | 25 | 134 |
| MRR20-FEM1-022008-A1 | 12 | 112 | 145 | 139 | 139 | 73 | 135 | 22 | 31 | 155 |
| MRR20-FEM1-022008-A3 | 5 | 121 | 143 | 149 | 140 | 118 | 128 | 23 | 26 | 143 |
| MRR20-FEM1-022008-A5 | 11 | 150 | 152 | 142 | 106 | 171 | 144 | 26 | 47 | 148 |
| MRU01-FEM1-022208-A1 | 6 | 141 | 165 | 169 | 173 | 122 | 137 | 24 | 32 | 183 |
| MRU01-FEM1-022208-A2 | 1c | 192 | 191 | 191 | 167 | 223 | 183 | 21 | 26 | 205 |
| MRU01-FEM1-022208-A3 | 1b | 109 | 136 | 133 | 171 | 107 | 110 | 22 | 27 | 142 |
| Mean | | 134.3 | 151.3 | 151.1 | 147.4 | 124.3 | 119.6 | 20.4 | 28.3 | 156.4 |
| STD | | 23.5 | 17.5 | 17.9 | 21.8 | 43.9 | 28.1 | 3.1 | 6.5 | 20.9 |
| CV | | 18% | 12% | 12% | 15% | 35% | 23% | 15% | 23% | 13% |

**Low numbers represent dark tones; high numbers represent light tones, gray values range from 0 to 255.*

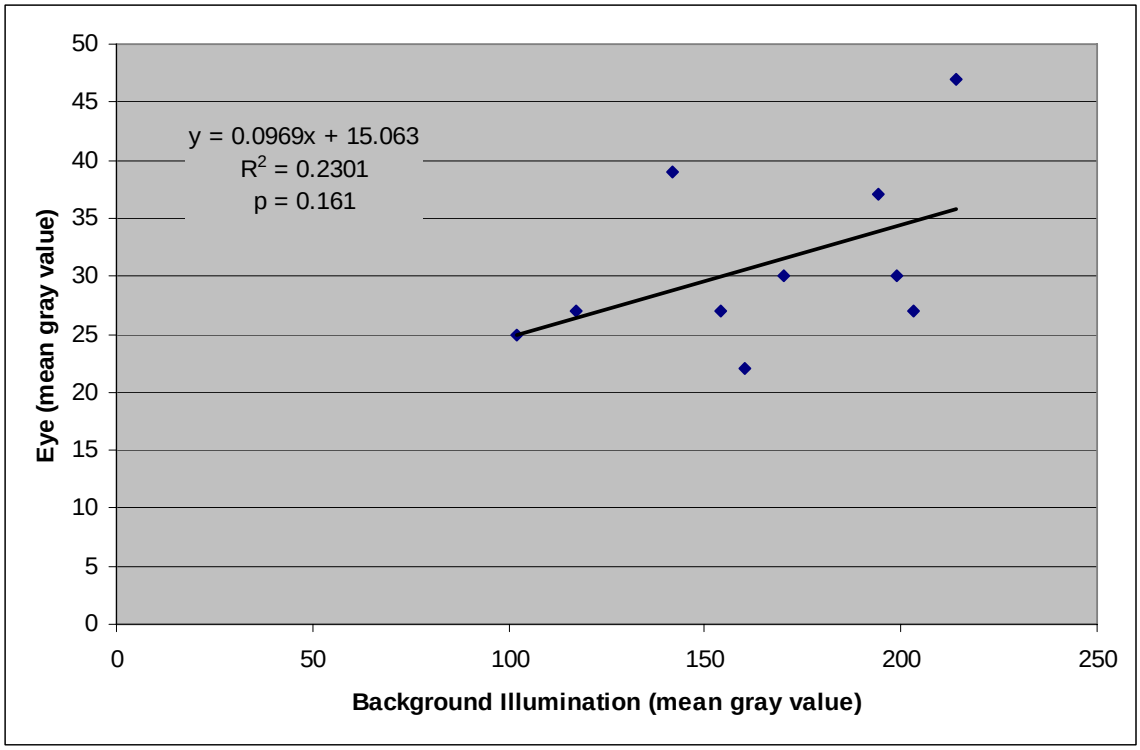



A. NATURALLY SPAWNED EMBRYOS

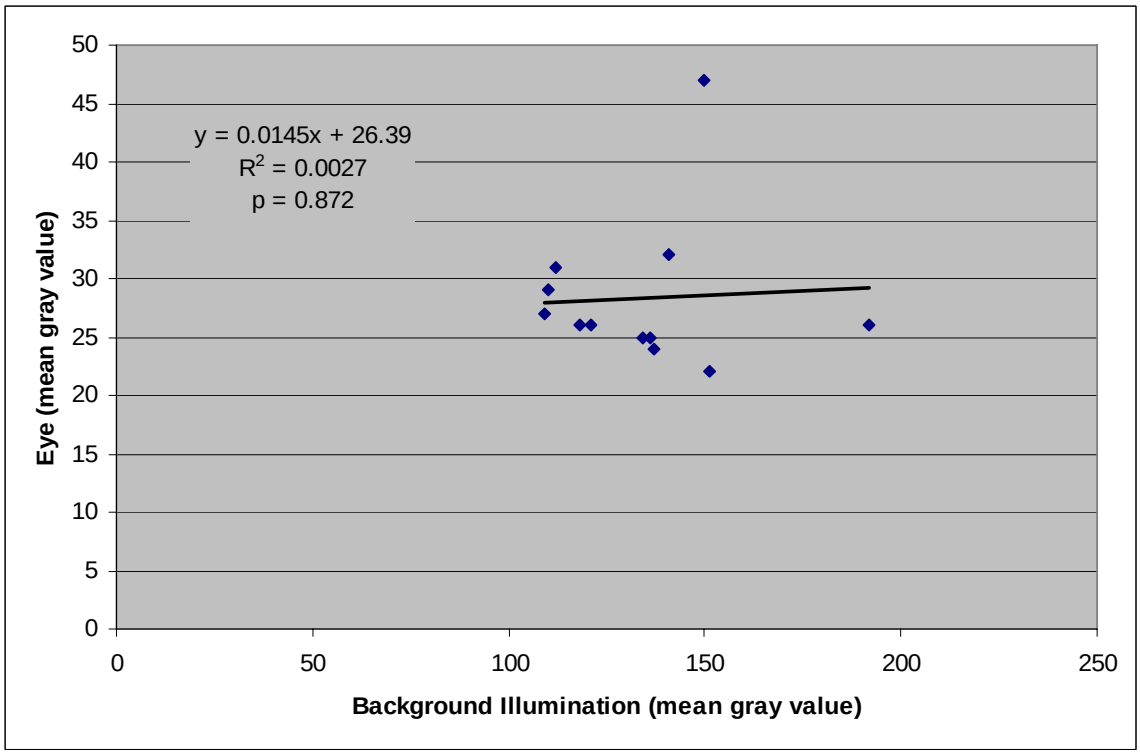



B. ARTIFICIALLY SPAWNED EMBRYOS

**FIGURE 16. Comparison of Background Illumination and Otic Vesicle**

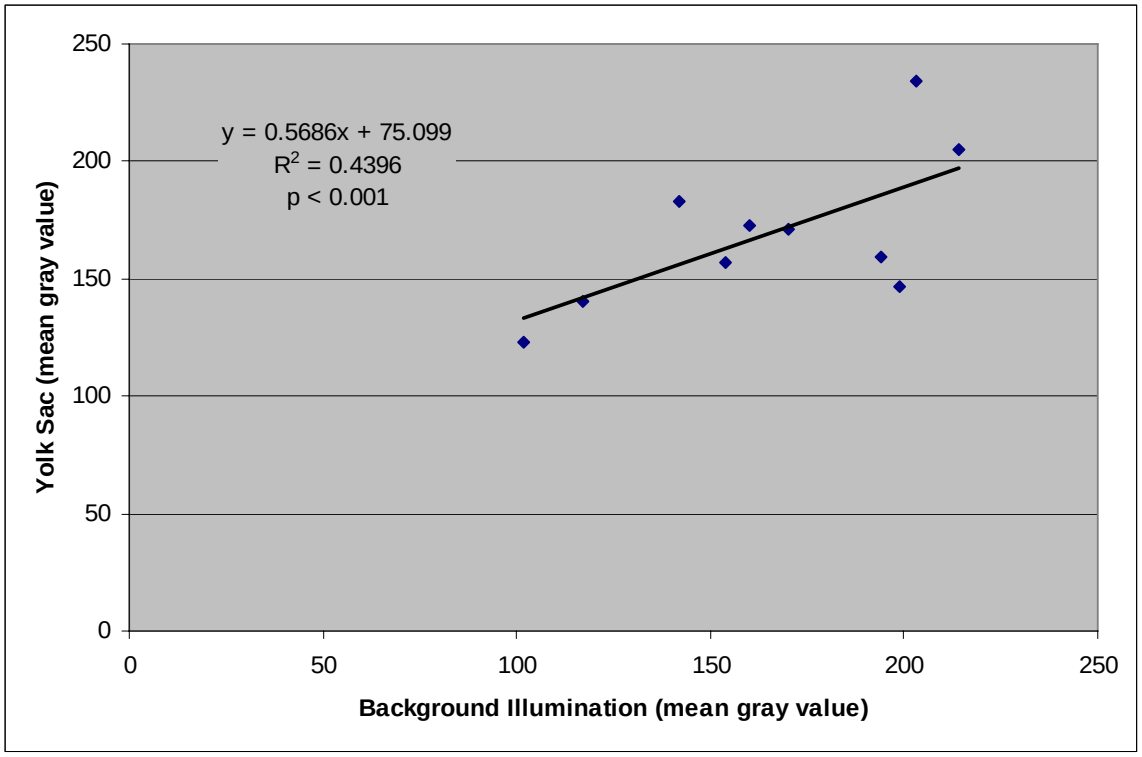



**A. NATURALLY SPAWNED EMBRYOS**

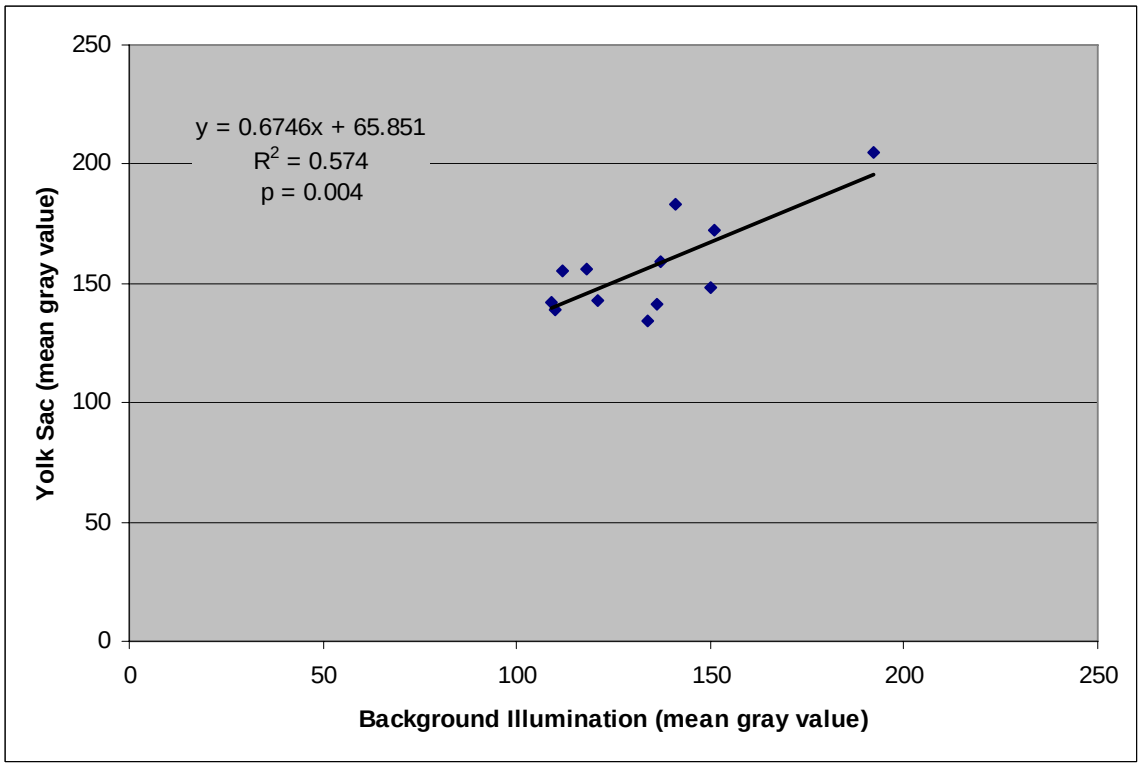



**B. ARTIFICIALLY SPAWNED EMBRYOS**

**FIGURE 17. Comparison of Background Illumination and Eye Pigmentation**




**A. NATURALLY SPAWNED EMBRYOS**




**B. ARTIFICIALLY SPAWNED EMBRYOS**

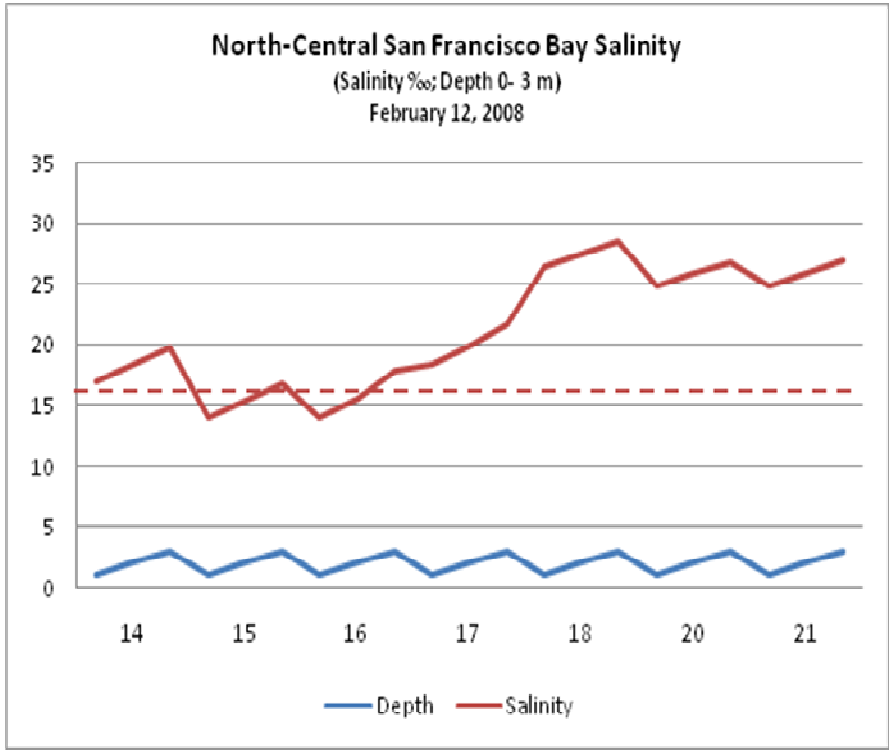
**FIGURE 18. Comparison of Background Illumination and Yolk Sac**

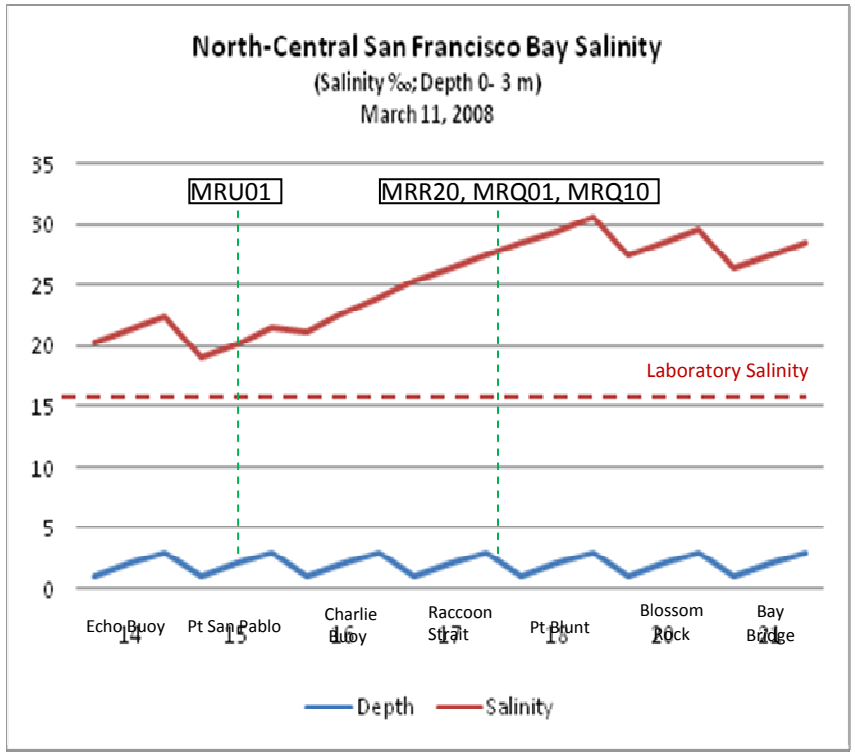
## Relationship of Qualitative Descriptions of Oiling, Local Salinity and Observations of Effects

Evaluations were made relative to the rank order of qualitative estimates of oiling provided (NOAA, BML, 2008). A summary of the relationships is presented in Table 8. Figure 19 portrays the salinities that were observed at nearby surface water stations during the spawning events. Figures 20 and 21 illustrate linear regression relationships among the various measurements with naturally and artificially spawned dechorionated embryos and degree of oiling present at the site locations. These regressions are built around average responses of the assessed embryos for each of the parameters based on 3 to 4 points of comparison. Caution is advised relative to the degree of confidence that can be placed in the observations. The majority of regression relationships have a low $R^2$ and the slopes are insignificant (i.e., not different from a horizontal line) indicating a lack of linear correlation between the parameter and the rank order of estimated oiling.

**TABLE 8. Summary of Significance of Regression Relationships between Natural and Artificial Spawn Data**

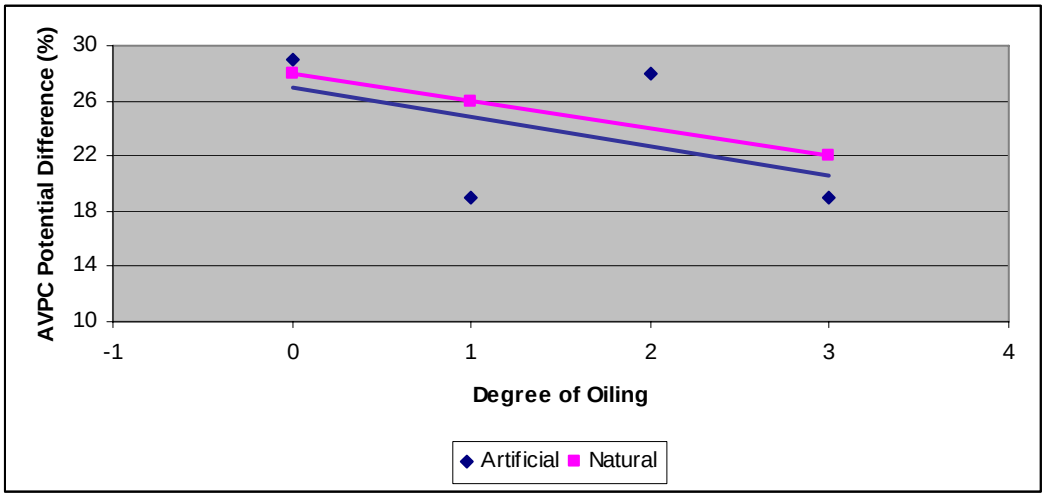
| Measurement | Natural (n=3) | | | Artificial (n=4) | | |
|---|---|---|---|---|---|---|
| | Slope Direction | Prob > F | $R^2$ | Slope Direction | Prob > F | $R^2$ |
| AVPC Potential % Difference | Neg | <0.001 | 1.0 | Neg | 0.507 | 0.243 |
| Yolk flattening (%) | Pos | 0.160 | 0.938 | Neg | 0.670 | 0.109 |
| Heart Rate | Neg | 0.971 | 0.001 | Pos | 0.938 | 0.004 |
| Heart Area | Neg | 0.429 | 0.611 | Neg | 0.325 | 0.456 |
| Pericardial Region | Pos | 0.927 | 0.015 | Neg | 0.599 | 0.161 |




*Data from http://sfbay.wr.usgs.gov/access/wqdata*

**Figure 19. Salinity Profiles for Central San Francisco Bay, February and March 2008**



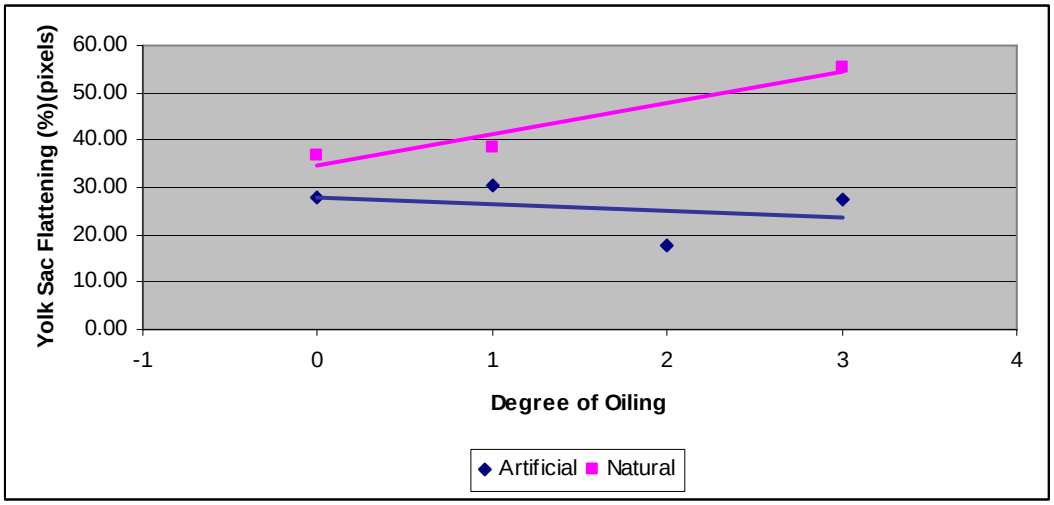




**Figure 20. Relationships between AVPC Length Data and Yolk Sac Flattening and Degree of Oiling**

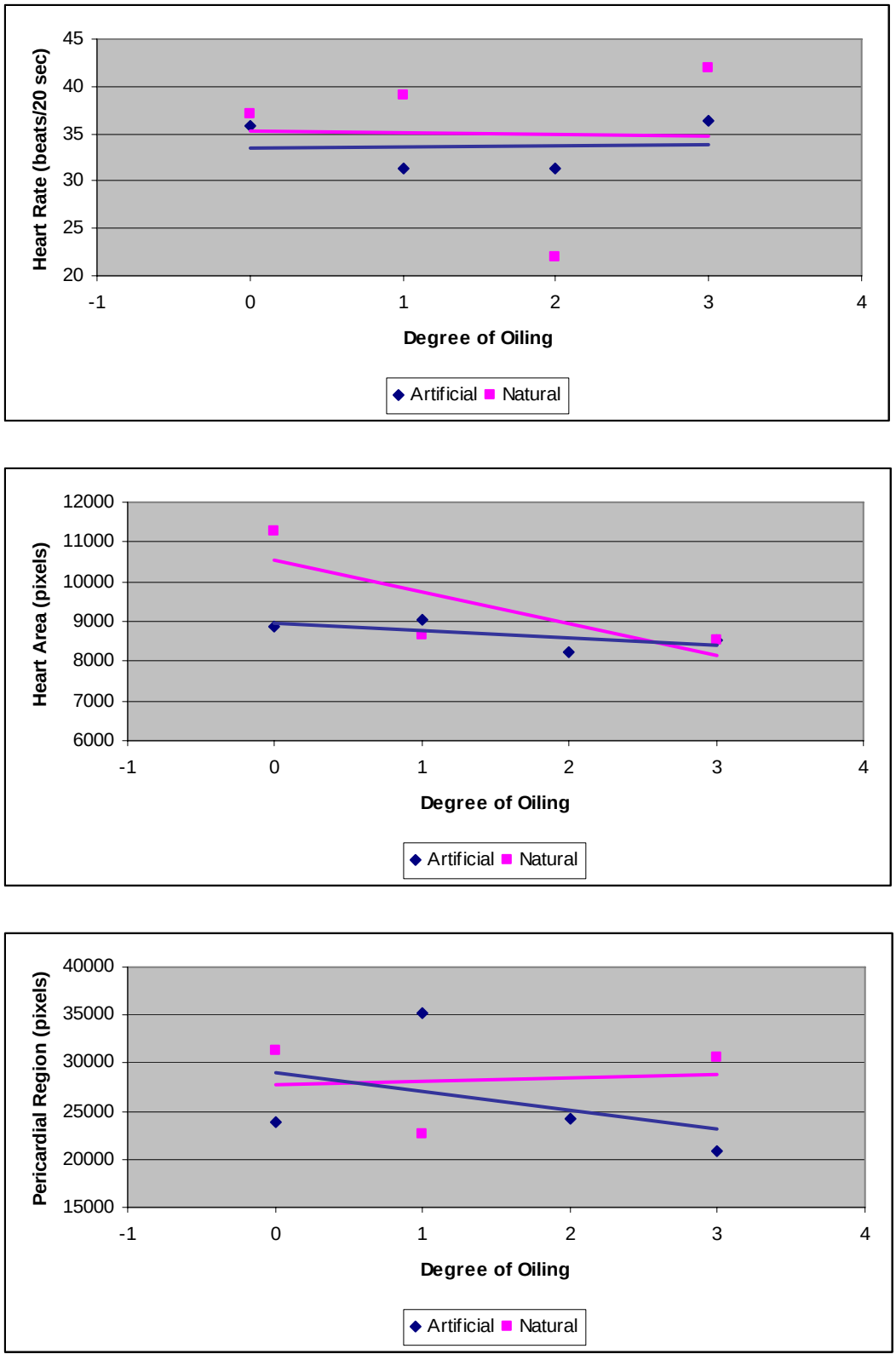



**Figure 21. Relationship between Heart Rate, Heart Area, and Pericardial Area and Degree of Oiling**

## Discussion

There is a distinct difference in the appearance of herring larvae from those organisms that were artificially spawned and developed at selected shallow subtidal locations and those that were spawned and developed naturally in nearshore, intertidal areas. The artificially spawned organisms are a known age and they have several differences between how they were handled compared to the naturally spawned embryos. The artificially spawned organisms were provided from harvest of adult herring selected for the larger sized individuals whereas the age structure of the adult herring comprising the Central San Francisco stock has not been evaluated at this time. The gonads used for the artificial spawning were harvested and fertilized at temperatures and salinities that were controlled (12°C and 16 ‰) and all organisms were spawned and collected on the artificial cages at the same time. Naturally spawned organisms were collected from areas with very different salinities ranging from salinities of ~16 - 28‰ (Figure 19). All organisms whether they were artificially or naturally spawned were exposed to seawater salinities of 16 ‰ during the dechorionation process. The artificially spawned embryos were subjected to scoring based upon video imaging of high quality while the naturally spawned embryos were primarily scored based on still images.

Several factors appear to be related to the observations collected on a subset of artificially and naturally spawned samples from four different locations. These factors probably had an impact on the assessment of oiling related impacts on Pacific herring in San Francisco Bay and need to be addressed in any future assessments. These factors include:

1. The location and environmental conditions of the water during spawning, development and hatching events.
2. The influence of environmental (salinity) and human factors (oiling) on the selected measurement end-points and how those influences effect assessments.
3. The stage of embryonic development when the fish were sampled and from which the measurements were made.
4. The consistency of the embryonic development stage upon which the measurements were made. The artificially spawned embryos are all one stage while the natural spawn are variable in developmental stage.
5. The illumination levels are variable and influence the density of pigmentation on various assessments of color and tissue density.
6. The age of the parental stocks and sizes of parents influence the size of eggs, development of embryos after fertilization and success of hatching. The age of parent stocks for naturally spawned embryos are unknown but the artificially spawned organisms were selected from larger individuals that were captured to provide the gametes used during this part of the project.

A key to understanding the effects of petroleum exposure on juvenile herring in San Francisco Bay requires a method to distinguish between the environmental and biological variables within the Bay that are also concordant with the rank order of oiling. Hershberger et al (2005) has demonstrated that younger adults have greater incidence of abnormal development in terms of notochord development, body shape and survival and successful development of the juveniles. The State of California's Herring Advisory Committee recognized various potential contributing

factors to the low population size of San Francisco Bay Herring stocks ranging from age structure (few 3-5 year fish), selective harvest of older fish, loss of habitat and the potential effects of contamination, including oil spills (California Fish & Game Commission 2008). The combination of many of these factors in addition to larger scale climate change has been attributed to be the cause(s) of the past decades historically low spawning biomass of herring in San Francisco Bay (SFEI Report). Since the characteristics being evaluated in this review are single measurements that may indicate responses to a variety of biological and chemical factors, it becomes important to evaluate factors which may be affecting the viability of natural larvae such as the age structure of the spawning populations. The winter of 2008 was different than many of the prior years with herring spawning in areas outside of their normal spawning sites. Cal Fish and Game regularly monitors the population structure of herring stocks in San Francisco Bay and these data should be available for examination to determine the size and age structure of the spawning stocks within the area of study as well as areas outside of the area of concern. The objective would be to compare the age structure of the stocks to data provided by Herschberger et al (2005) to determine if the age of the population of herring might have an influence on successful hatching.

The difference in handling of artificial and natural spawning events is an important element that needs to be addressed in any subsequent work. It is well known that herring can develop under a wide range of environmental salinities. Experimental laboratory protocols have demonstrated the wide range of tolerance to salinity and temperatures (Dinnel, et al. 2008). However, these protocols have been developed for determining success rates of fertilization, development, and hatching of larvae under consistent salinities throughout testing. The conditions associated with the natural and artificially spawned herring are different than these standardized protocols and would require appropriate acclimation to different salinities that occurred at points of capture and the subsequent laboratory trials for this study. Acclimation between the fertilization/development salinities and the water during hatching does not appear to have been addressed.

The potential and probable issue associated with this difference is built around the process of fertilization and early egg development. During the initial process of fertilization (after the sperm enters the egg) the eggs of fish imbibe the surrounding water and 'harden'. This captured water has its own characteristics and the egg is protected during development somewhat from the surrounding environment. Upon hatching, the larvae are then exposed to the surrounding environment. Under normal environmental conditions the ambient seawater at fertilization and at hatching should be relatively similar except in the intertidal under varying conditions of rainfall where local events alter this pattern and may explain some of the large variation in survival of intertidally spawned herring.

Standard technical procedures used in aquatic toxicology testing laboratories require slow acclimation of broodstock fishes over a period of at least two days to different salinities (ASTM E 1241). This slow acclimation over a period of at least two days and holding and developing the brood stock under these conditions during the collection and development of fertilized eggs assures minimum stress on the adults and developing eggs. The procedures outlined by Dinnel et al. (2008) follow this process as did the artificial spawning group during fertilization and dechorionation, although the development phase (post fertilization and hardening) was performed at environmental ambient conditions. The naturally spawned and developed eggs occurred in ambient water at the site but all of them were dechorionated in 16‰ water. It is anticipated that the larger the difference in the salinity of the imbibed water relative to the 'hatching' water used

during dechorionation the greater the stress to the newly 'hatched' individuals. The difference in salinity during spawning for the naturally spawned eggs is likely to result in osmotic gradients with water diffusing into tissues from the lower salinity environment during dechorionation. It is possible that the observations of extra membranes within the pericardial area, swelling of the pericardial area and flattening of the yolk sac during swelling could be related to the process just described. Edema created by this process or by petroleum exposures is unlikely to be distinguishable. The shapes and viability of the hatched/dechorionated larvae is also a characteristic that could result from being placed into different salinity environments. This is a key issue that needs to be addressed and incorporated into testing guidelines and protocols prior to doing future testing. An experiment to determine the influence of this process should be performed in order to eliminate this potential cause of any of the observations that were made in 2008 and that are proposed for 2009. This can be performed on a surrogate egg laying species (e.g., *Menidia beryllina*) prior to the herring spawning events in order to best establish a protocol for application to the herring study in 2009.

The last attribute that needs to be controlled is the amount of illumination transmitted to the viewing screens. An analysis of video images indicated that the density of pigmentation in the eye and otic vesicle were directly correlated to the amount of illumination measured by background grayness. Because of this factor the comments about pigmentation amounts is not supportable at this time. Future work will need to control this variable before significant conclusions can be made.

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# APPENDIX A

## A-1 SUMMARY OF AVAILABLE VIDEO AND STILL IMAGES

| File Descriptor | # Still Images | # Video Clips | Quick Assessment |
|---|---|---|---|
| MRP04-FEM1-021708-A1 | 32 | 36 | Usable |
| MRP04-FEM1-021708-A2 | 30 | 34 | Usable |
| MRP04-FEM1-021708-A3 | 30 | 37 | Usable |
| MRP04-FEM1-021708-A4 | 30 | 34 | Usable |
| MRP04-FEM1-021708-A5 | 30 | 34 | Usable |
| MRQ01-FEM1-021808-A1 | 31 | 31 | Usable |
| MRQ01-FEM1-021808-A2 | 31 | 31 | Usable |
| MRQ01-FEM1-021808-A3 | 30 | 31 | Usable |
| MRQ01-FEM1-021808-A4 | 30 | 30 | Poor, very pixilated screen captures, no video |
| MRQ01-FEM1-022908-N1 | 20 | 20 | Immature animals |
| MRQ01-FEM1-022908-N2 | 20 | 21 | Poor quality; immature animals, various planar views |
| MRQ01-FEM1-022908-N3 | 23 | 21 | Poor quality; immature animals, various planar views |
| MRQ01-FEM1-022908-N4 | 20 | 20 | Poor quality; immature animals, various planar views |
| MRQ01-FEM1-022908-N5 | 20 | 20 | Poor quality; immature animals, various planar views |
| MRQ01-FEM1-022908-N6 | 20 | 21 | Poor quality; immature animals; Clip 22 good image |
| MRQ01-FEM1-022908-N7 | 20 | 22 | Poor quality; immature animals, various planar views |
| MRQ01-FEM1-022908-N8 | 20 | 20 | Poor quality; immature animals, various planar views |
| MRQ10/P01-FEM1-021908-A1 | 31 | 31 | Usable |
| MRQ10/P01-FEM1-021908-A2 | 30 | 32 | Usable |
| MRQ10/P01-FEM1-021908-A3 | 31 | 33 | Usable |
| MRQ10 P01-FEM1-021808-A4 | 31 | 31 | Poor, very pixilated screen captures, no video |
| MRQ10/P01-FEM1-021908-A5 | 30 | 32 | Usable |
| MRQ10 P01-FEM1-022708-N1 | 27 | 21 | Poor quality; immature animals, various planar views |
| MRQ10 P01-FEM1-022708-N2 | 21 | 21 | Poor quality; immature animals, various planar views |
| MRQ10 P01-FEM1-022708-N3 | 22 | 21 | Poor quality; immature animals, various planar views |
| MRQ10 P01-FEM1-022708-N4 | 19 | 20 | Poor quality; immature animals, various planar views |
| MRQ10 P01-FEM1-022708-N5 | 27 | 20 | Poor quality; immature animals, various planar views |
| MRQ10 P01-FEM1-022708-N6 | 20 | 20 | Poor quality; immature animals, various planar views; Clips 6 and 20 possibly good video |
| MRQ10 P01-FEM1-022708-N7 | 19 | 21 | Usable |
| MRQ10 P01-FEM1-022708-N8 | 21 | 22 | Usable |
| MRR20-FEM1-022008-A1 | 31 | 33 | Usable |
| MRR20-FEM1-022008-A2 | 29 | 33 | Usable |
| MRR20-FEM1-022008-A3 | 30 | 31 | Usable |
| MRR20-FEM1-022008-A4 | 28 | 30 | Usable |
| MRR20-FEM1-022008-A5 | 30 | 30 | Usable |
| MRR20-FEM1-022808-N1 | 22 | 20 | Poor quality; immature animals, various planar views |
| MRR20-FEM1-022808-N2 | 26 | 22 | Poor quality; immature animals, various planar views |

## SUMMARY OF AVAILABLE VIDEO AND STILL IMAGES *(CONTINUED)*

| | | | |
|---|---|---|---|
| MRR20-FEM1-022808-N3 | 19 | 22 | Poor quality; immature animals, various planar views |
| MRR20-FEM1-022808-N4 | 26 | 23 | Poor quality; immature animals, various planar views; there are a few that may be usable |
| MRR20-FEM1-022808-N5 | 20 | 19 | Videos do not appear to be usable; have indistinct heart imaging |
| MRR20-FEM1-022808-N6 | 19 | 21 | Videos do not appear to be usable; have indistinct heart imaging |
| MRR20-FEM1-022808-N7 | 21 | 20 | Videos do not appear to be usable; have indistinct heart imaging |
| MRR20-FEM1-022808-N8 | 22 | 19 | Videos do not appear to be usable; have indistinct heart imaging |
| MRT04-FEM1-022508-A1 | 30 | 31 | Usable |
| MRT04-FEM1-022508-A2 | 30 | 35 | Usable; clip 30 is a good heart image |
| MRT04-FEM1-022508-A3 | 30 | 32 | Usable; clip 11 shows truncated pericardial region |
| MRT04-FEM1-022508-A4 | 32 | 35 | Usable; clip 17 irregular shaped yolk sac |
| MRT04-FEM1-022508-A5 | 30 | 30 | Usable |
| MRU01-FEM1-022208-A1 | 30 | 30 | Usable |
| MRU01-FEM1-022208-A3 | 30 | 30 | Usable |
| MRU01-FEM1-022208-A2 | 30 | 33 | Usable |
| MRU01-FEM1-022208-A4 | 29 | 32 | Usable; note may need to increase contrast |
| MRU01-FEM1-022608-N1 | 29 | 21 | Usable; clip 20 may have been over exposed? |
| MRU01-FEM1-022608-N2 | 26 | 20 | Usable |
| MRU01-FEM1-022608-N3 | 30 | 21 | Usable |
| MRU01-FEM1-022608-N4 | 26 | 20 | Some are usable but most do not clearly show heart; several images (clip 16, 21 show possible differences in pericardial region |
| MRU01-FEM1-022608-N5 | 24 | 20 | Usable |
| MRU01-FEM1-022608-N6 | 22 | 24 | Usable; CLIP 23 undeveloped jaw |
| MRU01-FEM1-022608-N7 | 30 | 20 | Usable; Clip 14: elongated pericardial region |
| MRU01-FEM1-022608-N8 | 26 | 20 | Usable; Clip 7, 4 compared to Clip 14: variation, elongation of pericardial region (how much variation is natural? Is this a developmental artifact? |
| whleggMRQ01-FEM1-022908-N3 | 6 | | images show process of dechorionation |
| Total Number | 1579 | 1565 | |
| # Stills for Natural Spawn (excluding MRP and MRT) | 727 | | |
| # Stills for Artificial Spawn (excluding MRP and MRT) | 542 | | |
| # Usable video | | 1109 | |

## A-2 SUMMARY OF NEWFIELDS REVIEW

| Parameters Assessed | Artificial Spawn *( #Assessed)* | | Natural Spawn *( #Assessed)* | |
|---|---|---|---|---|
| | MRQ10/P01 FEM1 021908 A1 (3), A2 (3), A3 (1), A5 (3) | | MRQ01 FEM1 022908 N3 (3), N6 (3); | |
| | MRU01 FEM1 022208 A1 (3), A2 (4), A3 (3) | | MRQ10 P01 FEM1 022708 N2 (3), N6 (3), N8 (3); | |
| | MRQ01 FEM1 021808 A1 (3), A3 (3); | | MRR20 FEM1 022808 N4 (3), N7 (3) | |
| | MRR20 FEM1 022008 A1 (3), A 3 (3), A 4 (3) | | MRU01 FEM1 022608 N2 (3), N4(3), N7(3) | |
| | Still Images | Video Clips | Still Images | Video Clips |
| Number Assessed (Status) | Previewed only | 35/243 | Preview only; detailed analysis of 10 samples | 30/208 |
| Development (qualitative) | x | | x | |
| Body Axis Alignment | | x | x * | x |
| Appearance | | x | x | x |
| Degree of Coiling: | | | x | |
| Eye Pigmentation | | x | | x |
| Development of Jaw | | x | | x |
| Yolk Sac Descriptors | | x | | x |
| Notochord | | x | | x |
| Body Pigmentation | | x | | x |
| Heart Structure | | x | | x |
| Heart Performance | | x | | x |
| Pericardial Region/Area | | x | | |
| AV Measurement | | x | | |
| Development of Otic Vesicle | | x | | x |
| Background Illumination (qualitative) | | | x | x |
| Background Illumination (quantitative) | | x | | x |
| Other Comments | | x | | x |

x* = *all still images (727)*

# A-3 COMMENTARY FROM VIDEO ANALYSES: ARTIFICIAL SPAWN
## MRQ01

| Site | Video Clip # | Morphology of Specimen | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Heart Structure | Heart Rate | Pericardial Region | Cranial Region | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| MRQ01- A1 | 4 | Appears to have different orientation, atrium appears rounded (top view); ventricle appears enlarged | 32 in 20 sec | Heart occupies 17% of the pericardial region | Otic vesicle has differentiation with two otoliths present; notochord has faint segmentation | 85% | Jaw notch present, jaw not well developed | Yolk sac is flattened, rounded not pigmented | 6/31, Clip 4 is flattened | |
| | 15 | Normal, backward L | 31 beats per 20 sec | Heart occupies 40% of the pericardial region | Otic vesicle has differentiation with two otoliths present; notochord is not segmented; darkened | 85% | Jaw notch present, jaw not well developed | Rounded, pigmented | | |
| | 28 | Normal, backward L | 28 beats in 20 second period | Heart occupies 32% of the pericardial region | Otic vesicle has differentiation with two otoliths present, but very heavily pigmented; segmentation of notochord not discernible | 85% | Jaw notch present, jaw developing | Rounded | | |
| MRQ01--A3 | 8 | Normal, backward L | 33 beats in 20 sec | Appears to be membrane developed which extends to heart; heart occupies 37% of pericardial region | Otic vesicle has differentiation with two otoliths present; segmentation of notochord | 85% | Jaw notch present, jaw developing noticeably | | | |
| | 13 | Normal, backward L, anterior/posterior extension | 34 beats in 20 sec | Extended posterior/anterior; heart occupies 51% of pericardial region | Head is not bent, linear; well defined otic vesicle; very faint segmentation of notochord | 85% | Very developed | Elliptical | | |
| | 30 | Normal, backward L | 30 beats in 20 sec | Heart occupies 44% of the pericardial region | Otic vesicle has differentiation with two otoliths present; notochord is not segmented | 85% | Very developed | Rounded, flattened on anterior side | Yes | |

## COMMENTARY FROM VIDEO ANALYSES: ARTIFICIAL SPAWN
## MRQ10

| Site | Video Clip # | Morphology of Specimen | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Heart Structure | Heart Rate | Pericardial Region | Cranial Region | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| MRQ10-A1 | 1 | Normal in shape backward L shape with atrium most dominant, ventricle elongated | 36 beats in a 20 second period; pattern was consistent | Heart occupies approximately 18% of pericardial region | Less highly differentiated | Eye appears to be colored | Jaw is not fully developed; no protrusion | | | Pericardium occupies a larger space than clip 5. This may be related to developmental age or possibly edema ; sample appears to be highly pigmented and granular in appearance |
| | 5 | Normal in shape backward L shape with atrium most dominant, ventricle elongated | 33 beats in a 20 second period; steady beat | Heart occupies 39% of pericardial region | Less highly differentiated; notochord not clearly segmented. | Eye appears to be colored | Jaw is not fully developed; notch developing on anterior side | Round in nature and heart is overlapped by yolk sac | | Pericardium may be more developed than the organism shown in Clip 1 based on the heart filling more of the pericardial space and the eye size tends to be smaller than the one in clip one. Image is darker making differentiation more difficult. Dorsum above notochord looks granulated. |
| | 19 | Normal in shape backward L shape with atrium most dominant, ventricle elongated ; ventral triangle anterior of yolk sac with septum | 32 beats in 20 second period | Heart occupies 36% of pericardial region; a more defined membrane bridging the ventral pericardial area to the yolk sac | Highly differentiated | Eye appears to be colored | Jaw is starting to become more pronounced | Normal in appearance | | Granulated yolk sac |
| MRQ10-A2 | 3 | | 28 beats in 20 second period | Heart occupies 19% of the pericardial region | Well defined; notochord not segmented | >85%; appears colored | Jaw is not fully developed; no protrusion | Yolk sac appears rounded; resembles stage q development | | |
| | 18 | | 30 beats in 20 second period | Heart occupies 29% of the pericardial region | Well defined; notochord not segmented | >85%; appears colored | Jaw not fully developed; no protrusion- anterior notch | Yolk sac appears rounded; resembles stage q development | | |
| | 26 | | 30 beats in 20 second period | Heart occupies 29% of the pericardial region | Well defined; notochord not segmented | >85%; appears colored | Jaw not fully developed; no protrusion | Yolk sac appears rounded; resembles stage q development | | |

## COMMENTARY FROM VIDEO ANALYSES: ARTIFICIAL SPAWN
## MRQ10 - *Continued*

| Site | Video Clip # | Morphology of Specimen | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Heart Structure | Heart Rate | Pericardial Region | Cranial Region | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| MRQ10-A3 | 29 | | 34 beats in 20 seconds | Heart occupies 28% of the pericardial region | Obvious segmentation in the notochord | 85% pigmentation | Jaw notch present, jaw not well developed | Rounded | | |
| MRQ10-A5 | 1 | Heart has a pinch on the ventral side when it is at the bottom of the down beat otherwise backward L; goes into the yolk sac on anterior side | 30 beats in 20 second period | Heart occupies 27% of the pericardial region | Otic vesicle has differentiation with two otoliths present; notochord is not segmented | 85% eye pigmentation | Jaw notch present, jaw not well developed | Yolk sac is intermediate between round and elliptical and appears to be granulated | | |
| | 8 | | 30 beats in 20 second period | Heart occupies 24% of the pericardial region | Otic vesicle has differentiation with two otoliths present; notochord has faint segmentation | 85% eye pigmentation | Jaw notch present, jaw not well developed | Yolk sac is intermediate between round and elliptical and appears to be granulated | | |
| | 20 | Appears to have a different orientation , atrial side is bulbous and extends beneath the yolk sac | 30 beats in 20 second period | Heart occupies 28% of the pericardial region which appears to be compressed than other images | Otic vesicle has differentiation with two otoliths present; notochord is not segmented | 85% eye pigmentation | Jaw is not developed but a notch is forming | Yolk sac is rounded and pushes into the pericardial region definitive and yolk sac is granulated | | Pigmentation on dorsal side of head |

## COMMENTARY FROM VIDEO ANALYSES: ARTIFICIAL SPAWN
## MRR20

| Site | Video Clip # | Morphology of Specimen | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Heart Structure | Heart Rate | Pericardial Region | Cranial Region | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| MRR20-FEM1-022008-A1 | 12 | Not normal, extremely compressed pericardial region | 24 beats per 20 sec | Completely abnormal, heart intruding into yolk sac or vice versa; Not measured | Not well defined; pigmentation | <50% eye pigmentation | Evident, but not protruded, and seems out of normal position | Flattened | | |
| | 25 | Y- shaped; atrium is on top of yolk sac | 29 beats per 20 sec | Heart occupies 29% of the pericardial region | Otic vesicle has differentiation with two otoliths present; notochord has faint segmentation | 60% eye pigmentation | Jaw notch present | Rounded, pigmented | No | |
| | 32 | Y-shaped; atrium is on top of yolk sac | 33 beats per 20 sec | Heart occupies 43% of the pericardial region | Otic vesicle has differentiation with two otoliths present; segmentation of notochord | 60% eye pigmentation | Jaw notch present | Rounded; slight flattening | No | |
| MRR20-FEM1-022008-A3 | 5 | Backwards L, with atrial intrusion into yolk sac area | 39 beats per 19 sec; major shutter at end of video (animal moved out of frame) | Pigmented; secondary pericardial membrane evident; heart occupies 38% of pericardial region | Otic vesicle has differentiation with two otoliths and semi-circular canals present dorsally and ventrally; notochord has faint segmentation; pigmentation | <85% | Developing jaw notch, intermediate protrusion | Rounded, flattened anterior side | | |
| | 19 | Normal backwards L | 42 beats per 20 sec | Heart occupies 37% of pericardial region | Differentiated, otoliths developed, branching semi-circular canals; notochord faintly visible | 60% | Developing jaw notch, protrusion | Elliptical | | |
| | 26 | Tipped backwards L; heart and yolk region overlaps | 37 beats per 20 sec | Heart occupies 37% of pericardial region | Heavy pigmentation; indistinct features in otic vesicle, notochord faintly segmented | <50 %; Eyes appear large compared to head region | Rudimentary notch present | Elliptical | | |

## COMMENTARY FROM VIDEO ANALYSES: ARTIFICIAL SPAWN
## MRR20 - *Continued*

| Site | Video Clip # | Morphology of Specimen | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Heart Structure | Heart Rate | Pericardial Region | Cranial Region | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| MRR20-FEM1-022008-A5 | 11 | Backward L, with atrial intrusion into yolk sac area | 41 beats per 20 sec | | Rudimentary development with faint segmentation of notochord; organism more transparent than most | ND | Rudimentary notch present | Elliptical; 6/30 | Yes | |
| | 17 | Very compressed; shape not clearly visible, very elongated | 45 beats per 20 sec | Very Compressed; | Rudimentary development, w/ 2 otoliths; faint segmentation of notochord; organism more transparent than most | 20% | Transparent, rudimentary, slight notch | Elliptical, flattened | Yes | |
| | 24 | Very compressed; shape visible; Backward L; atrium overlapping yolk sac | 37 beats in 20 sec | Very Compressed; | Rudimentary development with hardly any segmentation or the notochord | 30% | Early stage of notch formation | Rounded, flattened | yes | |

## COMMENTARY FROM VIDEO ANALYSES: ARTIFICIAL SPAWN
## MRU01

| Site | Video Clip # | Morphology of Specimen | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Heart Structure | Heart Rate | Pericardial Region | Cranial Region | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| MRU01-FEM1-022208-A1 | 6 | Normal in shape backward L shape with atrium most dominant, ventricle elongated | 36 beats in a 20 second period; pattern was consistent | Heart occupies approximately 37 % of pericardial region | Highly differentiated | 85% pigmented | Jaw is starting to develop with a notch | Yolk sac is elliptical | | Specimen has a pigmented granular appearance |
| | 18 | Abnormal in shape; V-shape, horizontal plane and extends into the yolk sac | 33 beats in a 20 second period; steady beat | Heart occupies approximately 46% of pericardial region; | Highly differentiated; notochord segmentation is not obvious | >85% pigmented | Jaw is not fully developed; notch starting to developing on anterior side | | | Specimen has a pigmented granular appearance |
| | 22 | Normal in shape backward L shape with atrium bulbous appearance, ventricle elongated | 32 beats in 20 second period | Heart occupies approximately 38% of pericardial region; | Highly differentiated; some segmentation of the notochord | Colored eye | Jaw well developed and starting to protrude | Elliptical shape | | Specimen has a pigmented granular appearance |
| MRU01-FEM1-022208-A3 | 1 | | 42 beats in 20 second period | Heart occupies 41% (measured) of the pericardial region | Well defined | ~85% pigmentation | Jaw is starting to become more pronounced | Yolk sac is elliptical (checked with elliptical tool | | |
| | 14 | | 38 beats in 20 second period | Heart occupies approximately 33% (measured) of the pericardial region | Well defined | > 85% colored | Jaw very pronounced | | | |
| | 21 | | 39 beats in 20 second period | Heart occupies 35% (measured) of the pericardial region; heart has v-shape aspect rather than backwards L | Well defined | ~85% pigmentation | Jaw very pronounced | | | Ventral edge of pericardial region not smooth arc with jaw protrusion; some pigmentation |

## COMMENTARY FROM VIDEO ANALYSES: ARTIFICIAL SPAWN
## MRU01 - *Continued*

| Site | Video Clip # | Morphology of Specimen | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Heart Structure | Heart Rate | Pericardial Region | Cranial Region | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| MRU01-FEM1-022208-A2 | 1 | | 31 beats in 20 second period | Heart occupies 33% (measured) of the pericardial region | Difficult to evaluate due to lighting; notochord segmentation evident | ~85% pigmentation | Jaw is starting to protrude | Yolk sac is elliptical | | Very light specimen, difficult to perceive depth |
| | 8 | | 35 beats in 20 second period | Heart occupies 34% (measured) of the pericardial region; heart protrudes into yolk region | Difficult to evaluate due to lighting; notochord segmentation evident | > 85% colored | Jaw is starting to protrude | Yolk sac is intermediate; more flattened appearance on pericardial side | | |
| | 10 | | 34 beats in 20 second period | Heart occupies 46% (measured) of the pericardial region; | Difficult to evaluate due to lighting; notochord segmentation not as developed | > 85% colored | Jaw notch is forming | Yolk sac is rounded | | |
| | 17 | | 39 beats in 20 second period | Heart occupies 33% (measured) of the pericardial region; atrial side is bulbous in shape | Well defined; segmentation of notochord very well defined (more than clip 10) | > 85% colored | Jaw notch is well formed | Yolk sac is elliptical | | |

# A-4 RESULTS FROM ARTIFICIAL SPAWN VIDEO ANALYSIS
## MRQ01

| Site | Video Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Potential % Length Difference | Heart Rate / 20sec | Heart Area | Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes/No | # in Sample | Freq |
| **MRQ01-FEM1-021808-A1** | | | | | | | | | | | | 6 | 31 | | 0 | 3 | |
| | 4 | | Minimum | Clip 4 Downbeat jpg | -119.8 | 134.8 | 9.9% | 32 | 5973 | 35196 | 17% | yes | | | no | | |
| | 4 | | Maximum | Clip 4 Downbeat jpg | 110.1 | 148.1 | 9.0% | | | | | | | | | | |
| | 15 | | Minimum | Clip 15 Upbeat jpg | 125.5 | 51.6 | 38.8% | 31 | 7867 | 19627 | 40% | no | | | no | | |
| | 15 | | Maximum | Clip 15 Upbeat jpg | -125.9 | 71.6 | 27.9% | | | | | | | | | | |
| | 28 | 27/618 | Minimum | Clip 28 Downbeat jpg | 108.7 | 59.1 | 35.4% | 28 | 9125 | 28743 | 32% | no | | | no | | |
| | 28 | | Maximum | Clip 28 Downbeat jpg | 108.2 | 80 | 26.1% | | | | | | | | | | |
| **Mean** | | | | | | | **24.5%** | **30.33** | **7,655.0** | **27,855.3** | **30%** | | | | | | |
| **MRQ01-FEM1-021808-A3** | | | | - | | | | | | | | 5 | 31 | | 1 | 3 | |
| | 8 | 48/622 | Minimum | Clip 8 Upbeat jpg | -103.2 | 48.3 | 36.6% | 33 | 10752 | 28734 | 37% | no | | | no | | |
| | 8 | | Maximum | Clip 8 Upbeat jpg | -103 | 66 | 26.8% | | | | | | | | | | |
| | 13 | 44/623 | Minimum | Clip 13 Downbeat jpg | 105.3 | 34.2 | 42.7% | 34 | 8167 | 15977 | 51% | no | | | no | | |
| | 13 | | Maximum | Clip 13 Downbeat jpg | -100.6 | 48.8 | 29.9% | | | | | | | | | | |
| | 30 | 135/619 | Minimum | Clip 30 Upbeat jpg | 109.9 | 50 | 37.2% | 30 | 7522 | 16954 | 44% | yes | | | yes | | |
| | 30 | | Maximum | Clip 30 Upbeat jpg | 108.7 | 68.6 | 27.1% | | | | | | | | | | |
| **Mean** | | | | | | | **33.4%** | **32.33** | **8,813.7** | **20,555.0** | **44%** | 11 | 62 | 18% | 1 | 6 | 17% |

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## RESULTS FROM ARTIFICIAL SPAWN VIDEO ANALYSES
## MRQ10

| Site | Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Potential % Length Difference | Heart Rate / 20sec | Heart Area | Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes | # in Sample | Freq |
| **MRQ10/P01-FEM1-021908-A1** | | | | | | | | | | | | 11 | 31 | | 0 | 3 | |
| | 1 | | Minimum | Clip 1 Upbeat SC | 69.3 | 113.3 | 29.6% | 36 | 9966 | 54305 | 18% | | | | | | |
| | 1 | | Maximum | Clip 1 Upbeat SC | 109.9 | 146.8 | 22.8% | | | | | | | | | | |
| | 5 | | Minimum | Clip 5 Downbeat SC | 104.9 | 93.1 | 15.1% | 33 | 8635 | 21986 | 39% | | | | | | |
| | 5 | | Maximum | Clip 5 Downbeat SC | 104 | 107.2 | 13.2% | | | | | | | | | | |
| | 19 | | Minimum | Clip 19 Downbeat SC | 72.5 | 79.7 | 32.1% | 32 | 9350 | 25879 | 36% | | | | | | |
| | 19 | | Maximum | Clip 19 Downbeat SC | 104.3 | 105.3 | 24.3% | | | | | | | | | | |
| Mean | | | | - | | | 22.9% | 33.7 | 9,317 | 34,057 | 31% | | | | | | |
| **MRQ10/P01-FEM1-021908-A2** | | | | | | | | | | | | 10 | 32 | | 0 | 3 | |
| | 3 | | Minimum | Clip 3 Upbeat SC | 74.5 | 134.9 | 22.6% | 28 | 8842 | 46591 | 19% | | | | | | |
| | 3 | | Maximum | Clip 3 Upbeat SC | 104.7 | 165.4 | 18.4% | | | | | | | | | | |
| | 18 | | Minimum | Clip 18 Upbeat SC | 99.7 | 95.4 | 18.9% | 30 | 11108 | 38878 | 29% | | | | | | |
| | 18 | | Maximum | Clip 18 Upbeat SC | 99.1 | 113.4 | 15.9% | | | | | | | | | | |
| | 26 | | Minimum | Clip 26 Upbeat SC | 104.3 | 97 | 12.5% | 30 | 8896 | 38894 | 23% | | | | | | |
| | 26 | | Maximum | Clip 26 Upbeat SC | 103.8 | 109.1 | 11.1% | | | | | | | | | | |
| Mean | | | | - | | | 16.6% | 29.3 | 9,615.3 | 41,454.3 | 23% | | | | | | |
| **MRQ10/P01-FEM1-021908-A3** | | | | - | | | | | | | | 9 | 33 | | 0 | 1 | |
| | 29 | | Minimum | Clip 29 Downbeat SC | 105.4 | 105.8 | 16.6% | 34 | 9943 | 35114 | 28% | | | | | | |
| | 29 | | Maximum | Clip 29 Downbeat SC | 107 | 123.4 | 14.3% | | | | | | | | | | |
| Mean | | | | - | | | 15.4% | | | | | | | | | | |

## RESULTS FROM ARTIFICIAL SPAWN VIDEO ANALYSES
## MRQ10 - *Continued*

| Site | Video Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Potential % Length difference | Heart Rate / 20sec | Heart Area | Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes | # in Sample | Freq |
| **MRQ10/P01-FEM1-021908-A5** | | | | | | | | | | | | 9 | 32 | | 0 | 3 | |
| | 1b | | Minimum | Clip 1 Upbeat SC | 106.5 | 112.6 | 24.2% | 30 | 8570 | 31534 | 27% | | | | | | |
| | 1b | | Maximum | Clip 1 Upbeat SC | 106.6 | 139.8 | 19.5% | | | | | | | | | | |
| | 8 | | Minimum | Clip 8 Upbeat SC | 102.7 | 86.1 | 21.7% | 30 | 8490 | 35228 | 24% | | | | | | |
| | 8 | | Maximum | Clip 8 Upbeat SC | 104.4 | 104.8 | 17.8% | | | | | | | | | | |
| | 20 | | Minimum | Clip 20 Upbeat SC | 105.3 | 80.2 | 25.7% | 30 | 6603 | 23643 | 28% | | | | | | |
| | 20 | | Maximum | Clip 20 Upbeat SC | 110.2 | 100.8 | 20.4% | | | | | | | | | | |
| **Mean** | | | | | | | **21.5%** | **30.00** | **7,887.7** | **30,135.0** | **26%** | 39 | 128 | 30% | **0** | **10** | **0%** |

## RESULTS FROM ARTIFICIAL SPAWN VIDEO ANALYSIS
## MRR20

| Site | Video Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Potential % Length Difference | Heart Rate / 20sec | Heart Area | Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes/No | # in Sample | Freq |
| **MRR20-FEM1-022008-A1** | | | | | | | | | | | | 11 | 33 | | 0 | 3 | |
| | 12 | ND | Minimum | Not Measured | ND | ND | ND | 24 | ND | ND | ND | yes | | | no | | |
| | 25 | 26/616 | Minimum | Clip 25 Upbeat jpg | 117.6 | 77.8 | 24.2% | 29 | 6378 | 22227 | 29% | no | | | no | | |
| | 25 | | Maximum | Clip 25 Upbeat jpg | 117.1 | 96.6 | 19.5% | | | | | | | | | | |
| | 32 | | Minimum | Clip 32 Upbeat jpg | 131.1 | 62.4 | 29.5% | 33 | 8292 | 19217 | 43% | yes | | | no | | |
| | 32 | | Maximum | Clip 32 Upbeat jpg | 131 | 80.8 | 22.8% | | | | | | | | | | |
| Mean | | | | | | | 24.0% | 28.67 | 7,335.0 | 20,722.0 | 36% | | | | | | |
| **MRR20-FEM1-022008-A3** | | | | | | | | | | | | 9 | 31 | | 0 | 3 | |
| | 5 | | Minimum | Clip 5 Downbeat jpg | 70.6 | 90.1 | 4.2% | 39 | 8930 | 23503 | 38% | yes | | | | | |
| | 5 | | Maximum | Clip 5 Downbeat jpg | 71.4 | 93.9 | 4.0% | | | | | | | | | | |
| | 19 | 58/620 | Minimum | Clip 19 Upbeat jpg | 108.7 | 59.1 | 39.1% | 42 | 11145 | 30132 | 37% | no | | | | | |
| | 19 | | Maximum | Clip 19 Upbeat jpg | 108.4 | 82.2 | 28.1% | | | | | | | | | | |
| | 26 | 182/614 | Minimum | Clip 26 Upbeat jpg | 90.7 | 77 | 24.7% | 37 | 10788 | 29014 | 37% | no | | | | | |
| | 26 | | Maximum | Clip 26 Upbeat jpg | 90 | 96 | 19.8% | | | | | | | | | | |
| Mean | | | | | | | 20.0% | 39.33 | 10,288 | 27,549.7 | 37% | | | | | | |

## RESULTS FROM ARTIFICIAL SPAWN VIDEO ANALYSIS
## MRR20 - *Continued*

| Site | Video Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Potential % Length Difference | Heart Rate / 20sec | Heart Area | Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes/No | # in Sample | Freq |
| **MRR20-FEM1-022008-A5** | | | | | | | | | | | | 6 | 30 | | 3 | 3 | |
| | 11 | 137/609 | Minimum | Clip 11 Upbeat jpg | 48.2 | 51 | 39.0% | 41 | 9284 | 21837 | 43% | no | | | yes | | |
| | 11 | | Maximum | Clip 11 Upbeat jpg | 49.6 | 70.9 | 28.1% | | | | | | | | | | |
| | 17 | 223/611 | Minimum | Clip 17 | -123.1 | 109.8 | 0.0% | 45 | 5606 | 10861 | 52% | yes | | | yes | | |
| | 17 | | Maximum | Clip 17 | 123.1 | 109.8 | 0.0% | | | | | | | | | | |
| | 24 | 300/599 | Minimum | Clip 24 Upbeat jpg | 131.7 | 97.7 | 18.3% | 37 | 7748 | 10652 | 73% | yes | | | yes | | |
| | 24 | | Maximum | Clip 24 Upbeat jpg | -130.4 | 115.6 | 15.5% | | | | | | | | | | |
| **Mean** | | | | - | | | **16.8%** | **41.00** | **7,546.0** | **14,450.0** | **56%** | **26** | **94** | **28%** | **3** | **9** | **33%** |

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## RESULTS FROM ARTIFICIAL SPAWN VIDEO ANALYSIS
## MRU01

| Site | Video Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Potential % Length difference | Heart Rate / 20sec | Heart Area | Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes/No | # in Sample | Freq |
| **MRU01-FEM1-022208-A1** | | | | | | | | | | | | 11 | 30 | | 0 | 3 | |
| | 6 | | Minimum | Clip 6 Upbeat SC | 106.2 | 57.3 | 28.8% | 36 | 8653 | 23198 | 37% | | | | | | |
| | 6 | | Maximum | Clip 6 Upbeat SC | 105.7 | 73.8 | 22.4% | | | | | | | | | | |
| | 18 | | Minimum | Clip 18 Upbeat SC | 117.3 | 34.9 | 49.9% | 33 | 9298 | 20303 | 46% | | | | | | |
| | 18 | | Maximum | Clip 18 Upbeat SC | 116.7 | 52.3 | 33.3% | | | | | | | | | | |
| | 22 | | Minimum | Clip 22 Upbeat SC | 112.1 | 39.9 | 56.9% | 32 | 9666 | 25138 | 38% | | | | | | |
| | 22 | | Maximum | Clip 22 Upbeat SC | 109.6 | 62.6 | 36.3% | | | | | | | | | | |
| Mean | | | | - | | | 37.9% | 33.67 | 9,205.7 | 22,879.7 | 41% | | | | | | |
| **MRU01-FEM1-022208-A2** | | | | - | | | | | | | | 9 | 33 | | 0 | 4 | |
| | 1c | | Minimum | Clip 1c Upbeat SC | 114.7 | 40.7 | 35.6% | 31 | 5514 | 16737 | 33% | | | | | | |
| | 1c | | Maximum | Clip 1c Upbeat SC | 67.6 | 55.2 | 26.3% | | | | | | | | | | |
| | 8 | | Minimum | Clip 8 Downbeat SC | 107.9 | 64.5 | 18.3% | 35 | 4914 | 14576 | 34% | | | | | | |
| | 8 | | Maximum | Clip 8 Downbeat SC | 109.1 | 76.3 | 15.5% | | | | | | | | | | |
| | 10 | | Minimum | Clip 10 Downbeat SC | 121.1 | 67.6 | 35.7% | 34 | 10898 | 23493 | 46% | | | | | | |
| | 10 | | Maximum | Clip 10 Downbeat SC | 122.1 | 91.7 | 26.3% | | | | | | | | | | |
| | 17 | | Minimum | Clip 17 Upbeat SC | 111.4 | 50.3 | 36.4% | 39 | 9764 | 29864 | 33% | | | | | | |
| | 17 | | Maximum | Clip 17 Upbeat SC | 114 | 68.6 | 26.7% | | | | | | | | | | |
| Mean | | | | - | | | 26.5% | 36.00 | 8,525.3 | 22,644.3 | 38% | | | | | | |

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## RESULTS FROM ARTIFICIAL SPAWN VIDEO ANALYSIS
## MRU01 - *Continued*

| Site | Video Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Potential % Length difference | Heart Rate / 20sec | Heart Area | Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes/No | # in Sample | Freq |
| **MRU01-FEM1-022208-A3** | | | | | | | | | | | | 6 | 30 | 20% | 0 | 3 | |
| | 1b | | Minimum | Clip 1 Upbeat SC | -108.8 | 49.3 | 28.2% | 42 | 9672 | 23590 | 41% | | | | | | |
| | 1b | | Maximum | Clip 1 Upbeat SC | -108.4 | 63.2 | 22.0% | | | | | | | | | | |
| | 14 | | Minimum | Clip 14 Downbeat SC | -111.8 | 53.9 | 39.1% | 38 | 10283 | 31419 | 33% | | | | | | |
| | 14 | | Maximum | Clip 14 Downbeat SC | -111.1 | 75 | 28.1% | | | | | | | | | | |
| | 21 | | Minimum | Clip 21 Upbeat SC | 120.3 | 55.6 | 12.6% | 39 | 10192 | 29489 | 35% | | | | | | |
| | 21 | | Maximum | Clip 21 Upbeat SC | -118.6 | 62.6 | 11.2% | | | | | | | | | | |
| **Mean** | | | | | | | **23.5%** | **39.67** | **10,049** | **28,166.0** | **36%** | **26** | **93** | **28%** | **0** | **10** | **0%** |

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## A-5 COMMENTARY ON NATURAL SPAWN VIDEO ANALYSES
### MRQ01

| Site | Video Clip # | Morphology | | | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Planar View | Rotation from Lateral Plane | Heart Structure | Heart Rate | Pericardial Region | Development of Otic Vesicle | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| **MRQ01-N3** | 11 | Left side | | Abnormal | 11 beats/20 sec | Compressed | Rudimentary | <50% | NM | Rounded, heavily pigmented | | |
| | 12 | Left side | Yes | Not discernible | 29/27 sec ~22/20 | Compressed | Not discernible | <50% | Not present | Rounded, flattening, secondary membrane | | |
| | 17 | Left side ventral | Yes | Not discernible | 27/20 sec | Not discernible | No view | <50% | Not present | Rounded, flattening, granular appearance | | |
| **MRQ01-N6** | 9 | Left side | Yes | Not discernible | 31/20 sec | Compressed | No view | <50% | Not present | Rounded, heavily pigmented, secondary membrane | | |
| | 10 | Left side | Yes | Not discernible | 28/20 sec | Compressed | No view | NM | Not present | Rounded, heavily pigmented, granular appearance | | |
| | 22 | Left side ventral | Yes | Not discernible | 13/20 sec | Not discernible | No view | NM | Not present | Rounded, flattening of anterior side, secondary membrane | | |

## COMMENTARY ON NATURAL SPAWN VIDEO ANALYSES
## MRQ10

| Site | Video Clip # | Morphology | | | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Planar View | Rotation from Lateral Plane | Heart Structure | Heart Rate | Pericardial Region | Otic Vesicle | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| **MRQ10-N2** | 91 | Left side | No | Backward L | 35/20 sec | Compressed, overlaps with yolk sac | Yes with some definition | NM | Rudimentary | Rounded, flattening, granular appearance | | |
| | 100 | Left side | No | Backward L | 38/21 sec | Appears normal may be some slight swelling need to make measurements | Yes with definition | ~50% | Yes-slight protrusion | Intermediate between rounded and elliptical, somewhat granular in nature | | |
| | 101 | Left side | No | Backward L | 36/20 sec | Appears normal need to make measurements | Yes with definition | <50% | Yes with protrusion | Elliptical with granulation | | |
| **MRQ10-N6** | 10 | Left side | Yes | Backward L | 38/20 sec | Appears normal need to make measurements | Yes with definition | NM | Rudimentary | Rounded, flattening on anterior side | | |
| | 20 | Left side | No | Backward L | 48/20 sec | Appears normal may be some slight swelling need to make measurements | Yes with some definition | ~50% | Yes-slight protrusion | Rounded, some granulation, secondary measurement | | Segmented notochord |
| | 22 | Left side | No | Backward L | 44/20 sec | Compressed, overlaps with yolk sac | Yes with definition | NM | Yes-slight protrusion | Intermediate between rounded and elliptical, somewhat granular in nature | | Segmented notochord |
| **MRQ10-N8** | 5 | Left side | No | Backward L | 37/20 sec | Appears normal, measurements possible | Yes with definition | >50% | Yes-slight protrusion | Rounded | | Segmented notochord |
| | 15 | Left side | No | Backward L | 34/20 sec | Compressed, overlaps with yolk sac, measurements possible | Yes with definition | <50% | Yes with protrusion | Rounded | | |
| | 22 | Left side | No | Backward L | 40/20 sec | Appears normal need to take measurements | Rudimentary | <50% | Rudimentary | Rounded | | Segmented notochord, sample is washed out |

## COMMENTARY ON NATURAL SPAWN VIDEO ANALYSES
### MRR20

| Site | Video Clip # | Morphology | | | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Planar View | Rotation from Lateral Plane | Heart Structure | Heart Rate | Pericardial Region | Otic Vesicle | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| **MRR20- N4** | 9 | Left side | No | Backward L | 39/20 sec | Appears normal need to take measurements | Yes with definition; notochord very faint | ~50% | Yes-slight protrusion | Rounded | | |
| | 20 | Left side | Yes | Not discernible | 42/20 sec | Compressed; some overlap with yolk sac, may be a secondary membrane anterior of yolk sac in lower corner | Yes with some definition; notochord not discernible | NM | Yes-slight protrusion | Rounded, with some flattening anterior side of yolk sac | | |
| | 23 | Left side | No | Backward L | 50/21 sec | Appear elongated from head to yolk sac; ventricle bulbous; heart occupies left side of pericardial region, measurements possible | Rudimentary, some definition of notochord | 50% | Rudimentary | Only partially viewed in image | | Segmented notochord |
| **MRR20-N7** | 51 | Ventral | Yes | Not discernible | 38/20 sec | Not discernible | Rudimentary | <50% | Not present | Rounded with flattening of anterior edge, pigmented | | Specimen is very granular |
| | 57 | Left side | No | Not discernible | 50/20 sec | Elongated appearance, may have swollen pericardial, measurement not possible; secondary membrane is distinct and appears granulated | Yes with some definition | <50% | Yes-slight protrusion | Elliptical with a pinch on anterior side, double membrane | | Notochord with some segmentation, specimen has ghost-like appearance |
| | 66 | right side | Yes | Not discernible | 34/21 sec | Not discernible | Not discernible | Not discernible | Not discernible | Rounded with flattening of anterior edge, pigmented | | Pectoral fin is present on right side of pericardial region |

## COMMENTARY ON NATURAL SPAWN VIDEO ANALYSES
### MRU01

| Site | Video Clip # | Morphology | | | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Planar View | Rotation from Lateral Plane | Heart Structure | Heart Rate | Pericardial Region | Otic Vesicle | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| **MRU01-FEM1-022608-N2** | 3 | Left side | No | Backward L-attached to jaw region on the ventricle side | 42/20 sec | Appears normal, measurements possible | Yes with definition | Not discernible | Yes-intermediate protrusion | Rounded | | Notochord with segments, still images look good most are linear |
| | 14 | Left side | No | Backward L-attached to jaw region on the ventricle side | 35/20 sec | Appears normal, measurements possible, secondary membrane anterior of yolk sac | Yes with definition | Not discernible | Yes, intermediate protrusion | Rounded | | |
| | 19 | Left side | No | Backward L-attached to jaw region on the ventricle side | 34/20 sec | Appears normal, measurements possible | Yes with definition | Not discernible | Yes-slight protrusion | Rounded | | Image somewhat grainy and dark |
| **MRU01-FEM1-022608-N4** | 5 | Left side | No | Backward L-attached to jaw region on the ventricle side | 36/20 sec | Appears normal, measurements possible | Yes with some definition | Not discernible | Yes-slight protrusion | Rounded, with some flattening anterior side of yolk sac | | Notochord with segments, still images look good most are linear |
| | 11 | Left side | No | Y - laying on its side | 37/20 sec | Appears normal, measurements possible | Yes with definition | 85% | Yes with protrusion | Rounded | | Notochord with segments |
| | 14 | Left side | No | Backward L | 36/20 sec | Elongated appearance, may have swollen pericardial with extended secondary pericardial membrane evident, measurement possible | Yes with some definition | Not discernible | Yes-very protruded | Rounded with double membrane on ventral edge | | Notochord with some segmentation |

## COMMENTARY ON NATURAL SPAWN VIDEO ANALYSES
## MRU01- CONTINUED

| SIte | Video Clip # | Morphology | | | | | | | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Planar View | Rotation from Lateral Plane | Heart Structure | Heart Rate | Pericardial Region | Otic Vesicle | Eye Pigmentation | Jaw | Yolk Sac | Arrhythmia | |
| MRU01-FEM1-022608-N7 | 8 | Left side | No | Not discernible | 37/20 sec | Elongated appearance almost looks like two distinct pericardial regions, may have swollen pericardial, measurement possible | Yes with definition | <50% | Yes-very protruded, extends under eye | Appears rounded, only partially in view | | Notochord with some segmentation |
| | 16 | Left side | No | Backward L-attached to jaw region on the ventricle side | 37/20 sec | Heart occupies left side of pericardial region, measurements possible | Yes with definition | <50% | Yes-very protruded, extends under eye | Appears rounded, only partially in view | | Notochord with some segmentation, image has some granulation |
| | 21 | Left side | No | Backward L-attached to jaw region on the ventricle side | 40/20 sec | Elongated appearance almost looks like two distinct pericardial regions, secondary pericardial membrane evident, measurement possible | Yes with definition | ~50% | Yes-very protruded, extends under eye | Appears rounded with double membrane, only partially in view | | |

## A-6 RESULTS FROM NATURAL SPAWN VIDEO ANALYSES

### MRQ01

| Site | Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Difference Length (%) | Heart Rate / 20sec | Heart Area | Area Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes | # in Sample | Freq |
| MRQ01-FEM1-022908-N3 | | | | | | | | | | | | NM* | NM | NM | 0 | 3 | 0 |
| | 11 | | NM | NM | NM | NM | NM | 11 | | | NM | no | | | | | |
| | 12 | | NM | NM | NM | NM | NM | 22 | | | NM | yes | | | | | |
| | 17 | | NM | NM | NM | NM | NM | 27 | | | NM | yes | | | | | |
| MRQ01-FEM1-022908-N6 | | | | | | | | | | | | | | | | | |
| | 9 | | NM | NM | NM | NM | NM | 31 | | | NM | no | | | 0 | 3 | 0 |
| | 10 | | NM | NM | NM | NM | NM | 28 | | | NM | no | | | | | |
| | 22 | | NM | NM | NM | NM | NM | 13 | | | NM | yes | | | | | |
| **MRQ01 Mean** | | | | | | | | **22** | | | | **NM** | **NM** | **NM** | **0** | **3** | **0** |

## RESULTS FROM NATURAL SPAWN VIDEO ANALYSES
## MRQ10

| Site | Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Difference Length (%) | Heart Rate / 20sec | Heart Area | Area Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes | # in Sample | Freq |
| MRQ10/P01-FEM1-022708-N2 | | | | | | | | | | | | 9 | 21 | 43% | 1 | 3 | |
| | 91 | | NM | NM | NM | NM | NM | 35 | | | NM | yes | | | | | |
| | 100 | | Minimum | Clip 100 Upbeat jpg | 66.6 | 47.9 | 57% | 38 | 9811 | 24398 | 40% | no | | | | | |
| | 100 | | Maximum | Clip 100 Upbeat jpg | 109.9 | 146.8 | 67.4% | | | | | | | | | | |
| | 101 | 62/620 | Minimum | Clip 101 Upbeat jpg | 55.3 | 47.4 | 36.9% | 36 | 6464 | 20752 | 31% | yes | | | | | |
| | 101 | | Maximum | Clip 101 Upbeat jpg | 56.3 | 64.9 | 27.0% | | | | | | | | | | |
| MRQ10/P01-FEM1-022708-N6 | | | | | | | | | | | | 9 | 20 | 45% | 0 | 3 | |
| | 10 | | Minimum | Clip 10 Downbeat jpg | -78.3 | 78.6 | 19.7% | 38 | 7480 | 19338 | 39% | | | | | | |
| | 10 | | Maximum | Clip 10 Downbeat jpg | -77.7 | 94.1 | 16.5% | | | | | | | | | | |
| | 20 | 54/614 | Minimum | Clip 20 Upbeat jpg | 63 | 55 | 34.2% | 48 | 10342 | 31904 | 32% | | | | | | |
| | 20 | | Maximum | Clip 20 Upbeat jpg | 63.4 | 73.8 | 25.5% | | | | | | | | | | |
| | 22 | | Minimum | Clip 20 Upbeat jpg | 69.4 | 59.8 | 12.7% | 44 | 8947 | 18607 | 48% | | | | | | |
| | 22 | | Maximum | Clip 20 Upbeat jpg | 69.1 | 67.4 | 11.3% | | | | | | | | | | |

## RESULTS FROM NATURAL SPAWN VIDEO ANALYSES
## MRQ10 – *Continued*

| Site | Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Difference Length (%) | Heart Rate / 20sec | Heart Area | Area Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes | # in Sample | Freq |
| MRQ10/P01-FEM1-022708-N8 | | | | | | | | | | | | 6 | 22 | 27% | | | |
| | 5 | | Minimum | Clip 5 Upbeat | 95.1 | 45.2 | 40.0% | 37 | 10713 | 27950 | 38% | no | | | 0 | 3 | 0 |
| | 5 | 26/612 | Maximum | Clip 5 Upbeat | 95.4 | 63.3 | 28.6% | | | | | | | | | | |
| | 15 | | Minimum | Clip 15 - Upbeat | 112 | 50.7 | 30.8% | 34 | 8113 | 13612 | 60% | no | | | | | |
| | 15 | | Maximum | Clip 15 - Upbeat | 113.1 | 66.3 | 23.5% | | | | | | | | | | |
| | 22 | 23/621 | Minimum | Clip 22 - Downbeat | 100.8 | 47.9 | 11.5% | 40 | 7269 | 24852 | 29% | no | | | | | |
| | 22 | | Maximum | Clip 22 - Downbeat - | 77 | 53.4 | 10.3% | | | | | | | | | | |
| **MRQ10 Mean** | | | | | | **26.4%** | **37.6%** | **39** | **,642** | **22,677** | **40%** | **24** | **63** | **36%** | **1** | **9** | **11 %** |

## RESULTS FROM NATURAL SPAWN VIDEO ANALYSES
## MRR20

| Site | Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Difference Length (%) | Heart Rate / 20sec | Heart Area | Area Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes | # in Sample | Freq |
| MRR20-FEM1-022808-N4 | | | | | | | | | | | | NM | NM | NM | 0 | 3 | |
| | 79 | 100/678 | Minimum | Clip 9 Downbeat | 90 | 91 | 9.9% | 39 | 8194 | 30013 | 27% | no | | | | | |
| | 9 | | Maximum | Clip 9 Downbeat | 90 | 100 | 9.0% | | | | | | | | | | |
| | 20 | 161/622 | Minimum | Clip 20 Downbeat | 90 | 49 | 40.8% | 42 | 8913 | 26509 | 34% | yes | | | | | |
| | 20 | | Maximum | Clip 20 Downbeat | 90 | 69 | 29.0% | | | | | | | | | | |
| | 23 | 18/634 | Minimum | Clip 23 Upbeat | 58.2 | 49.4 | 24.7% | 48 | 8446 | 35472 | 24% | no | | | | | |
| | 23 | | Maximum | Clip 23 Upbeat | 57.6 | 61.6 | 19.8% | | | | | | | | | | |
| MRR20-FEM1-022808-N7 | | | | | | | | | | | | 10 | 18 | 56% | 2 | 3 | |
| | 51 | | Minimum | Clip 51 | NM | NM | NM | 38 | NM | NM | NM | | | | | | |
| | 51 | | Maximum | Clip 51 | NM | NM | NM | | NM | NM | | | | | | | |
| | 57 | | Minimum | Clip 57 | NM | NM | NM | 50 | NM | NM | NM | | | | | | |
| | 57 | | Maximum | Clip 57 | NM | NM | NM | | NM | NM | | | | | | | |
| | 66 | | Minimum | Clip 66 | NM | NM | NM | 34 | NM | NM | NM | | | | | | |
| | 66 | | Maximum | Clip 66 | NM | NM | NM | | NM | NM | | | | | | | |
| **MRR20 Mean** | | | | | | | **22.2%** | **41.8** | **8518** | **30665** | **28%** | **10** | **18** | **56%** | **0** | **6** | **33%** |

## RESULTS FROM NATURAL SPAWN VIDEO ANALYSES
## MRU01

| Site | Clip # | Frame | Position of AV Canal during Heartbeat | Record | Angle | Length (px) | Difference Length (%) | Heart Rate / 20sec | Heart Area | Area Pericardial Region | %Heart: Pericardium | Yolk Sac Flattening (Group Video Images) | | | Arrhythmia (Individual Video Images) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Yes | # in Sample | Freq | Yes | # in Sample | Freq |
| MRU01-FEM1-022608-N2 | | | | | | | | | | | | 8 | 20 | 40% | 0 | 3 | |
| | 3 | 9/616 | Minimum | Clip 3 Upbeat | 112 | 50.7 | 32.9% | 42 | 12744 | 29101 | 44% | no | | | | | |
| | 3 | | Maximum | Clip 3 Upbeat | 110.9 | 67.4 | 24.8% | | | | | | | | | | |
| | 14 | 57/604 | Minimum | Clip 14 Downbeat | 114.9 | 45.2 | 38.7% | 35 | 11691 | 19242 | 61% | no | | | | | |
| | 14 | | Maximum | Clip 14 Downbeat | 66.3 | 62.7 | 27.9% | | | | | | | | | | |
| | 19 | 111/607 | Minimum | Clip 19 Downbeat | 90 | 41 | 39.0% | 34 | 11950 | 21157 | 56% | no | | | | | |
| | 19 | | Maximum | Clip 19 Downbeat | 90 | 57 | 28.1% | | | | | | | | | | |
| MRU01-FEM1-022608-N4 | | | | | | | | | | | | 7 | 20 | 35% | 0 | 3 | |
| | 5 | 17/621 | Minimum | Clip 5 Upbeat | 92.7 | 63.1 | 28.4% | 36 | 11395 | 42087 | 27% | yes | | | | | |
| | 5 | | Maximum | Clip 5 Upbeat | 90 | 81 | 22.1% | | | | | | | | | | |
| | 11 | 38/614 | Minimum | Clip 11 Upbeat | 98.1 | 49.5 | 36.8% | 37 | 11378 | 34116 | 33% | | | | | | |
| | 11 | | Maximum | Clip 11 Upbeat | 102.8 | 67.7 | 26.9% | | | | | | | | | | |
| | 14 | 45/610 | Minimum | Clip 14 Upbeat | 90 | 45 | 15.6% | 36 | 11457 | 37783 | 30% | | | | | | |
| | 14 | | Maximum | Clip 14 Upbeat | 88.9 | 52 | 13.5% | | | | | | | | | | |
| MRU01-FEM1-022608-N7 | | | | | | | | | | | | 7 | 20 | 35% | 1 | 3 | |
| | 8 | 31/608 | Minimum | Clip 8 Upbeat | 90 | 44 | 29.5% | 37 | 10692 | 35770 | 30% | | | | | | |
| | 8 | | Maximum | Clip 8 Upbeat | 90 | 57 | 22.8% | | | | | | | | | | |
| | 16 | 23/612 | Minimum | Clip 16 Upbeat | 90 | 36 | 44.4% | 37 | 8063 | 30769 | 26% | | | | | | |
| | 16 | | Maximum | Clip 16 Upbeat | 90 | 52 | 30.8% | | | | | | | | | | |
| | 21 | 12/615 | Minimum | Clip 21 Upbeat | 90 | 59 | 30.5% | 40 | 12048 | 31898 | 38% | | | | | | |
| | 21 | | Maximum | Clip 21 Upbeat | 90 | 77 | 23.4% | | | | | | | | | | |
| **MRU01 Mean** | | | | | | | **28.7%** | **37** | **11,269** | **31,325** | **38.4%** | **22** | **60** | **36.7%** | **1** | **9** | **11%** |

## A-7 ILLUMINATION ASSESSMENT - ARTIFICIAL SPAWN (SUBSET OF VIDEO FILES)

**Artificial Spawn**

| Site | Clip # | Illumination *(mean gray value*)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Background | | | Head | | | Eye | | Yolk Sac |
| | | Top Left | Bottom Right | Bottom Left | Dorsal | Otic Vesicle | Dorsal, 2nd | Center | Outside | Not in Shadow |
| MRQ01-FEM1-021808-A1 | 4 | 137 | 153 | 153 | 155 | 139 | 100 | 17 | 24 | 159 |
| MRQ01-FEM1-021808-A3 | 8 | 136 | 129 | 135 | 116 | 117 | 105 | 17 | 25 | 141 |
| MRQ10/P01-FEM1-021908-A1 | 1 | 151 | 174 | 174 | 131 | 148 | 85 | 20 | 22 | 172 |
| MRQ10/P01-FEM1-021908-A2 | 3 | 110 | 140 | 137 | 149 | 71 | 107 | 17 | 29 | 139 |
| MRQ10/P01-FEM1-021908-A3 | 29 | 118 | 141 | 145 | 154 | 73 | 82 | 17 | 26 | 156 |
| MRQ10/P01-FEM1-021908-A5 | 1b | 134 | 146 | 146 | 168 | 129 | 119 | 19 | 25 | 134 |
| MRR20-FEM1-022008-A1 | 12 | 112 | 145 | 139 | 139 | 73 | 135 | 22 | 31 | 155 |
| MRR20-FEM1-022008-A3 | 5 | 121 | 143 | 149 | 140 | 118 | 128 | 23 | 26 | 143 |
| MRR20-FEM1-022008-A5 | 11 | 150 | 152 | 142 | 106 | 171 | 144 | 26 | 47 | 148 |
| MRU01-FEM1-022208-A1 | 6 | 141 | 165 | 169 | 173 | 122 | 137 | 24 | 32 | 183 |
| MRU01-FEM1-022208-A2 | 1c | 192 | 191 | 191 | 167 | 223 | 183 | 21 | 26 | 205 |
| MRU01-FEM1-022208-A3 | 1b | 109 | 136 | 133 | 171 | 107 | 110 | 22 | 27 | 142 |

**low numbers = dark tones (dark orange color); high numbers = light tones (light orange color)*

## ILLUMINATION ASSESSMENT - NATURAL SPAWN (SUBSET OF VIDEO FILES)

**Natural Spawn**

| Site | Clip # | Illumination *(mean gray value*)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Background | | | Head | | | Eye | | Yolk Sac |
| | | Top Left | Bottom Rt | Bottom Left | Dorsal | Otic Vesicle | Dorsal, 2nd | Center | Outside | Not in Shadow |
| MRQ01-FEM1-022908-N4 | 11 | 194 | 210 | 206 | 118 | 154 | 146 | 27 | 37 | 159 |
| MRQ01-FEM1-022908-N6 | 6 | 199 | 201 | 206 | 134 | 151 | 132 | 22 | 30 | 147 |
| MRQ10/P01-FEM1-022708-N2 | 91 | 142 | 154 | 155 | 162 | 114 | 114 | 33 | 39 | 183 |
| MRQ10/P01-FEM1-022708-N6 | 10 | 170 | 186 | 188 | 198 | 190 | 105 | 20 | 30 | 171 |
| MRQ10/P01-FEM1-022708-N8 | 5 | 154 | 161 | 128 | 211 | 160 | 130 | 21 | 27 | 157 |
| MRR20-FEM1-022808-N4 | 9 | 203 | 209 | 216 | 241 | 159 | 149 | 22 | 27 | 234 |
| MRR20-FEM1-022808-N7 | 51 | 214 | 217 | 210 | 135 | 91 | 129 | 34 | 47 | 205 |
| MRU01-FEM1-022608-N2 | 3 | 102 | 128 | 126 | 121 | 60 | 78 | 22 | 25 | 123 |
| MRU01-FEM1-022608-N4 | 5 | 160 | 161 | 157 | 136 | 142 | 111 | 20 | 22 | 173 |
| MRU01-FEM1-022608-N7 | 8 | 117 | 145 | 138 | 172 | 66 | 201 | 22 | 27 | 140 |

**low numbers = dark tones (dark orange color); high numbers = light tones (light orange color)*

## A-8 ANALYSIS OF STILL IMAGES OF NATURALLY SPAWNED EMBRYOS FOR OCCURRENCE OF BODY AXIS IRREGULARITIES

### MRQ01

| Site | # Still Images | Quick Assessment | Appearance | Body Axis Defect | C | G | O | S | NM | Linear | Total # | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MRQ01-FEM1-022908-N1 | 20 | Immature animals | Gold tone present (19/20) | 9 | 1 | 1 | 3 | 2 | 3 | 1 | 20 | Linear specimens appear to have less gold than others |
| MRQ01-FEM1-022908-N2 | 20 | Poor quality; immature animals, various planar views | Gold tone present | 10 | | | 5 | 3 | 1 | 1 | 20 | Linear specimens appear to have less gold than others |
| MRQ01-FEM1-022908-N3 | 22 | Poor quality; immature animals, various planar views | Gold tone present | 13 | | 1 | 5 | 3 | | | 22 | Jpeg 9 &10 same image; 18 & 19 are same image |
| MRQ01-FEM1-022908-N4 | 20 | Poor quality; immature animals, various planar views | Gold tone present | 12 | | | 1 | 3 | 4 | | 20 | Background illumination is very dark and textured |
| MRQ01-FEM1-022908-N5 | 20 | Poor quality; immature animals, various planar views | Gold tone present | 9 | | 1 | 4 | 4 | 2 | | 20 | |
| MRQ01-FEM1-022908-N6 | 19 | Poor quality; immature animals; Clip 22 good image | Gold tone present | 9 | 1 | 1 | 2 | 6 | | | 19 | Background very white, washed out, textured |
| MRQ01-FEM1-022908-N7 | 20 | Poor quality; immature animals, various planar views | Gold tone present | 11 | | | | 1 | 8 | | 20 | Background illumination is very dark and textured |
| MRQ01-FEM1-022908-N8 | 20 | Poor quality; immature animals, various planar views | Gold tone present | 11 | | | | 1 | 8 | | 20 | |
| | | | **Total Number** | **84** | **2** | **4** | **20** | **23** | **26** | **2** | **161** | |

*Codes: C = c-shaped; G = g-shaped; O = o-shaped; S = s-shaped; NM = Not measurable; Linear = Normal horizontal orientation*

## ANALYSIS OF STILL IMAGES OF NATURALLY SPAWNED EMBRYOS FOR OCCURRENCE OF BODY AXIS IRREGULARITIES
## MRQ10

| Site | # Still Images | Quick Assessment | Appearance | Body Axis Defect | C | G | O | S | NM | Linear | Total # | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MRQ10 P01-FEM1-022708-N1 | 25 | Poor quality; immature animals, various planar views | Gold tone present | 18 | | 2 | | 1 | 3 | 1 | 25 | Repeated image 16 &17; linear specimen have less gold tone |
| MRQ10 P01-FEM1-022708-N2 | 21 | Poor quality; immature animals, various planar views | Gold tone present | 13 | 1 | | 2 | | 1 | 4 | 21 | Background illumination is very dark and textured |
| MRQ10 P01-FEM1-022708-N3 | 21 | Poor quality; immature animals, various planar views | Gold tone present | 13 | | | 2 | | | 6 | 21 | Repeat image 3&4, 18&19; background very dark |
| MRQ10 P01-FEM1-022708-N4 | 19 | Poor quality; immature animals, various planar views | Gold tone present | 19 | | | | | | | 19 | Background illumination is very dark and textured |
| MRQ10 P01-FEM1-022708-N5 | 21 | Poor quality; immature animals, various planar views | Gold tone present | 14 | 3 | | 1 | | 2 | 1 | 21 | At image 18, background illumination changes to lighter (very textured) |
| MRQ10 P01-FEM1-022708-N6 | 20 | Poor quality; immature animals, various planar views; Clips 6 and 20 possibly good video | Gold tone present | 16 | 1 | | | | 1 | 2 | 20 | Background illumination changes |
| MRQ10 P01-FEM1-022708-N7 | 18 | Usable | Gold tones not noticeable | 6 | | | | | 2 | 10 | 18 | Image 17 good of linear development; 1 image not usable |
| MRQ10 P01-FEM1-022708-N8 | 21 | Usable | Gold tones not noticeable | 8 | | | | 1 | | 12 | 21 | Washed out, white background |
| | | | **Total Number** | **107** | **5** | **2** | **5** | **2** | **9** | **36** | **166** | |

*Codes: C = c-shaped; G = g-shaped; O = o-shaped; S = s-shaped; NM = Not measurable; Linear = Normal horizontal orientation*

## ANALYSIS OF STILL IMAGES OF NATURALLY SPAWNED EMBRYOS FOR OCCURRENCE OF BODY AXIS IRREGULARITIES
## MRR20

| Site | # Still Images | Quick Assessment | Appearance | Body Axis Defect | C | G | O | S | NM | Linear | Total # | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MRR20-FEM1-022808-N1 | 22 | Poor quality; immature animals, various planar views | White background | 16 | 1 | | | | 2 | 3 | 22 | Various tones in background |
| MRR20-FEM1-022808-N2 | 21 | Poor quality; immature animals, various planar views | Gold tones, Varied dark to light | 19 | | | | | 1 | 1 | 21 | |
| MRR20-FEM1-022808-N3 | 19 | Poor quality; immature animals, various planar views | Gold tones, Varied dark to light | 19 | | | | | | | 19 | |
| MRR20-FEM1-022808-N4 | 21 | Poor quality; immature animals, various planar views; there are a few that may be usable | Washed out white; some gold tones when non-linear | 14 | | | | | 1 | 6 | 21 | Image 6 & 7 repeated; 8&9 repeated; 10&11 repeated; 23 &24 repeated; 25&26 repeated |
| MRR20-FEM1-022808-N5 | 20 | Videos do not appear to be usable; have indistinct heart imaging | Washed out white; gold tones | 15 | | | 3 | 1 | 1 | | 20 | |
| MRR20-FEM1-022808-N6 | 18 | Videos do not appear to be usable; have indistinct heart imaging | Washed out white; gold tones | 16 | | | 2 | | | | 18 | 18 & 19 repeated |
| MRR20-FEM1-022808-N7 | 21 | Videos do not appear to be usable; have indistinct heart imaging | Washed out white; gold tones | 10 | | 1 | 6 | 1 | 1 | 1 | 20 | 12&13 repeat |
| MRR20-FEM1-022808-N8 | 22 | Videos do not appear to be usable; have indistinct heart imaging | Washed out white; gold tones | 13 | | 1 | 4 | 3 | 1 | | 22 | |
| | | | **Total Number** | **122** | **1** | **2** | **15** | **5** | **7** | **11** | **163** | |

*Codes: C = c-shaped; G = g-shaped; O = o-shaped; S = s-shaped; NM = Not measurable; Linear = Normal horizontal orientation*

## ANALYSIS OF STILL IMAGES OF NATURALLY SPAWNED EMBRYOS FOR OCCURRENCE OF BODY AXIS IRREGULARITIES
## MRU01

| Site | # Still Images | Quick Assessment | Appearance | Body Axis Defect | C | G | O | S | NM | Linear | Total # | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MRU01-FEM1-022608-N1 | 21 | Usable; clip 20 may have been over exposed? | Washed out white to gray; no gold tones | | | | | | | 21 | 21 | Image 2,3,4 are repeats; 17&18 same; 19&20 same image; 21&22 same; 23 &24 same; 26,27, 28 same image |
| MRU01-FEM1-022608-N2 | 20 | Usable | Mixed; Transparent white to gray (washed out white at bottom) | | | | | | | 20 | 20 | 2,3 same; 4,5 same; 6,7 same; 8,9 same; 10,11 same; 17,18 same |
| MRU01-FEM1-022608-N3 | 17 | Usable | White to gray background; no gold tone | | | | | | | 17 | 17 | 2,3 same; 4,5 same; 6,7,8 same; 9,10,11,12 same; 15,16, 17 same; 18,19 same; 21,22 same; 23,24 same; 28,29 same |
| MRU01-FEM1-022608-N4 | 19 | Some are usable but most do not clearly show heart; several images (clip 16, 21 show possible differences in pericardial region | washed out white; no gold tones | 5 | | | | | | 14 | 19 | 5,6 same; 7,8 same; 9,10 same; 11,12 same; 17,18 same; 20,21 same; 25,26 same |
| MRU01-FEM1-022608-N5 | 19 | Usable | Mixed; no gold tones | | 1 | | | | | 18 | 19 | 2,3 same; 11,12 same; 21,22 same; 23,24, 25 same |
| MRU01-FEM1-022608-N6 | 21 | Usable; CLIP 23 undeveloped jaw | Mixed; no gold tones | 4 | 3 | | | | | 14 | 21 | 12,13 same; 15,16 same; 17,18 same; 24, 25 same |
| MRU01-FEM1-022608-N7 | 21 | Usable; Clip 14: elongated pericardial region | Translucent, shades of gray | | | | | | | 21 | 21 | 6,7 same; 10,11 same; 12,13 same; 14,15 same; 16, 17 same; 19, 20 same; 22,23 same; 25, 26 same; 30,31 same |
| MRU01-FEM1-022608-N8 | 20 | Usable; Clip 7, 4 compared to Clip 14: variation, elongation of pericardial region (how much variation is natural? Is this a developmental artifact? | Translucent, shades of gray | 6 | 1 | | | | | 13 | 20 | 4,5 same; 11,12 same; 13,14 same; 15, 16 same; 20,21 same; 24, 25 same |
| | | | **Total Number** | **15** | **5** | **0** | **0** | **0** | **0** | **138** | **158** | |

*Codes: C = c-shaped; G = g-shaped; O = o-shaped; S = s-shaped; NM = Not measurable; Linear = Normal horizontal orientation*