Echocardiographic evaluation in koi carp (Cyprinus carpio) under manual restraint compared to anesthesia with isoeugenol

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OBJECTIVE
To establish an echocardiographic technique in koi carp (Cyprinus carpio), compare cardiopulmonary parameters under manual restraint versus anesthesia, and provide a gross anatomical and histologic cardiac description.

METHODS
A randomized, crossover echocardiography study was performed in 40 clinically healthy adult, unknown sex, privately owned koi carp on May 10 and 11 through June 26 and 27, 2021. Echocardiography was examined for each koi under manual restraint and isoeugenol at 50 ppm, with 3 measurements per examination performed by a radiologist and cardiologist. Two koi were euthanized for gross anatomic and histologic cardiac evaluation.

RESULTS
Mean ejection fraction (EF), stroke volume (SV), and cardiac output (CO) were significantly lower, mean heart rate (HR) was significantly higher, and opercular rate (OPR) was decreased significantly in anesthetized compared to manually restrained koi. Poor reproducibility for EF and SV was observed.

CONCLUSIONS
Echocardiography was feasible in both manually restrained and anesthetized koi; however, this technique may best be applied to monitoring trends over time in individual fish due to low reproducibility. Significant differences in multiple cardiopulmonary parameters, including HR, EF, SV, CO, and OPR, were present between manually restrained and anesthetized koi. A gross anatomical and histologic cardiac description is provided for this species to pair with the echocardiographic images.

CLINICAL RELEVANCE
This study provides the first description of echocardiography, cardiac gross anatomy, and histology in koi. The results support echocardiography as a safe and practical noninvasive diagnostic for cardiac assessment in koi under both manual restraint and anesthesia.

Keywords: anesthesia, Cyprinus carpio, isoeugenol, koi, echocardiograph
documented in a few fish species under varying condition, ranging from conscious to anesthetized, and has not yet been reported in koi carp (Cyprinus carpio). Koi are popular ornamental fish found worldwide as display animals in private collections, zoological institutions, and aquariums. Koi commonly present for veterinary evaluation that often requires anesthesia; however, the echocardiographic parameters and cardiac effects of anesthesia are unknown in the species, limiting diagnostic interpretation and presenting patient-monitoring challenges for the veterinarian.

Isoleugenol is an immersion anesthetic agent derived from clove oil used for sedation and anesthesia in fish. Studies in fish, including koi, have shown it to be an effective anesthetic due to the fast induction of anesthesia, short duration of recovery, rapid excretion, wide margin of safety, and lack of residues or harmful effects to fish or humans during use. A study in koi suggested isoeugenol at 40 to 100 ppm may be useful for both induction and maintenance of anesthesia. However, additional studies are indicated as bradycardia can lead to hypoxemia in fish species, and isoeugenol at 60 ppm has been shown to decrease heart rate (HR), cardiac output (CO), dorsal aortic pressure, and stroke volume (SV) in Chinook salmon (Oncorhynchus tshawytscha).

The objectives of this study were to establish a safe technique for echocardiographic evaluation in koi and compare cardiopulmonary parameters under manual restraint versus anesthesia with isoeugenol (AQUI-S; New Zealand Ltd) at 50 ppm. As an adjunct to the echocardiographic images, a secondary objective of the study was to provide a gross anatomical and histologic cardiac description of the koi heart. We hypothesized that cardiac parameters, including HR, SV, CO, and ejection fraction (EF), would be statistically different between the 2 groups and expected to see cardiovascular depression in the anesthetized group.

Methods

Animals and tanks

Forty clinically healthy, adult, unknown sex koi carp were maintained at a private koi facility in Watkinsville, Georgia. The koi were visually distinct due to their unique patterns and were photographed after the first study for identification purposes. The koi were housed indoors in a system of 2 15,000-gallon freshwater tanks. Each tank had a filtration system comprised of 10-cubic-foot prop-washed bead filters, 4-ton upflow oyster bed filters, 400-watt UV sterilizers, and a shower tower. The water quality in each tank was remotely monitored twice an hour by a wireless fidelity control unit for temperature, pH, ammonia, dissolved oxygen, and water level. General hardness and nitrite were tested (API Master Freshwater Testing Kit; Mars Fishcare) twice a week, and the tanks were maintained to the following parameters: temperature, 12.8 to 15.5°C; pH, 7.5; general hardness, 100 to 120; and dissolved oxygen, 6.5 to 8.0 mg/L. The koi were fed a commercial diet (Japan Pet Design Company) of 44% crude protein at a rate of 2% to 3% of their body weight per day. The tank water was sampled at the start of the study for routine water analysis, which was unremarkable.

Study design

The study methods and procedures were reviewed and approved by the University of Georgia’s Clinical Research Committee, and consent was obtained by the private owner of the koi. The experiment was designed and implemented as a crossover design, with n = 40 koi each assessed via echocardiography under 2 treatments: manual restraint and anesthesia. Fish were randomly assigned to 1 of 2 orders of treatment (manual restraint–anesthesia vs anesthesia–manual restraint), with 20 fish per order. The experiment sessions occurred first on May 10 and 11, 2021, followed by a 47-day washout period, then the second session on June 26 and 27, 2021. Opercular rate (OPR), HR, and echocardiogram were evaluated for each fish under both treatments. A total of 80 measurements for HR and OPR were analyzed (1 measurement for each of the 2 treatments for 40 fish). Each echocardiogram was assessed by 2 veterinarian observers, 1 radiologist and 1 cardiologist, to reduce measurement error and to assess reliability. Each observer measured each echocardiogram 3 times, and the mean of the 3 assessments for EF, SV, and CO was used for statistical analysis. A total of 160 observations for EF, SV, and CO were analyzed (1 for each of the 2 observers for each of the 2 treatments within each of the 40 fish).

Manual restraint and anesthesia

The koi were individually netted and transported to 1 of 2 smaller (100-cm length X 80-cm width) experimental tanks used for the echocardiograms. Manual restraint or anesthesia start time began upon entry of the koi into the experimental tank without an acclimation period. Snout-to-tail length (cm) was measured for each koi after entry into the experimental tank prior to starting the echocardiogram. Water from their original housing tanks was used for the experimental tank and repeatedly changed out throughout the study to ensure consistent water quality. A clear plastic bag, modified with an opening on both ends to allow water flow, was placed over the tank to reduce measurement error and to assess reliability. Each koi was equipped with a radiotelemetry tag to track movement of the koi during treatment. A total of 80 observations for EF, SV, and CO was used for statistical analysis.

Studies

Experiments in fish, including koi, have shown isoeugenol to be an effective anesthetic due to the fast induction and presenting patient-monitoring challenges for the veterinarian. A study in koi suggested isoeugenol at 40 to 100 ppm may be useful for both induction and maintenance of anesthesia. However, additional studies are indicated due to their unique patterns and were photographed after the first study for identification purposes. The koi were housed indoors in a system of 2 15,000-gallon freshwater tanks. Each tank had a filtration system comprised of 10-cubic-foot prop-washed bead filters, 4-ton upflow oyster bed filters, 400-watt UV sterilizers, and a shower tower. The water quality in each tank was remotely monitored twice an hour by a wireless fidelity control unit for temperature, pH, ammonia, dissolved oxygen, and water level. General hardness and nitrite were tested (API Master Freshwater Testing Kit; Mars Fishcare) twice a week, and the tanks were maintained to the following parameters: temperature, 12.8 to 15.5°C; pH, 7.5; general hardness, 100 to 120; and dissolved oxygen, 6.5 to 8.0 mg/L. The koi were fed a commercial diet (Japan Pet Design Company) of 44% crude protein at a rate of 2% to 3% of their body weight per day. The tank water was sampled at the start of the study for routine water analysis, which was unremarkable.

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Healthcare) with a 6- to 10-MHz microconvex ultrasound transducer, and cine loops were recorded and saved for postprocedure analysis. The long axis view was obtained by a ventral midline approach between the opercula as demonstrated in Figure 1. The probe was held in an oblique plane approximately 30 to 45 degrees from the midsagittal plane oriented in a left-cranial-to-right-caudal direction. The probe was rocked cranially or caudally to orient the left ventricular outflow tract at a 30- to 60-degree angle to the ultrasound beam when obtaining all Doppler images. The short axis view was obtained by rotating the probe 90 degrees from the long axis view in a counterclockwise direction. Color flow Doppler cine loops were acquired in long axis and short axis (Figure 2).

**Echocardiographic measurement**

The single-plane Simpson method of disks was utilized to assess ventricular volumes. The Simpson method of disks is the preferred method for measuring volume, which estimates cardiac volume as a summation of parallel cylinders, the diameters of which are derived from tracing the endocardial border of the left ventricle.\(^{16-18}\) The length of the ventricle is measured from the atrioventricular valve annulus to the ventricular apex, and the area is then divided into equally sized elliptical disks, the summation of which yields ventricular volume:

\[
V = \frac{\pi}{4} \sum_{i=1}^{n} a_i b_i
\]

where \(n\) = number of disks, \(l\) = ventricle length, and \(a\) and \(b\) = the diameter of the disks.\(^{16,18}\)

Cardiac cycles were assessed for optimal visualization of the endocardium. Measurements were performed on cardiac cycles that had the least amount of foreshortening with clear distinction of the endocardium. As an ECG was not available for timing the events of the cardiac cycle, end diastole and end systole were estimated by the frames that showed the heart at its maximum (diastole) and

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**Figure 1**—Echocardiographic evaluation in 40 clinically healthy adult, unknown sex, privately owned koi carp under manual restraint or isoeugenol at 50 ppm on May 10 and 11 and June 26 and 27, 2021. This image depicts handling under manual restraint and ultrasound probe placement in the long axis view for echocardiographic imaging of an adult koi carp utilizing a plastic barrier between fish and handler.

**Echocardiographic examination**

The echocardiographic examination was started once koi were adequately settled in position under manual restraint (ie, no excessive movement) or sufficiently anesthetized (ie, no response to handling). Opercular rate (beats per minute) was monitored throughout the procedure and recorded for all koi at the start of probe placement and at 5 minutes into the procedure. The echocardiographic examination was performed by a veterinary radiologist using a veterinary ultrasound system (GE Logiq E; GE Healthcare) with a 6- to 10-MHz microconvex ultrasound transducer, and cine loops were recorded and saved for postprocedure analysis. The long axis view was obtained by a ventral midline approach between the opercula as demonstrated in Figure 1. The probe was held in an oblique plane approximately 30 to 45 degrees from the midsagittal plane oriented in a left-cranial-to-right-caudal direction. The probe was rocked cranially or caudally to orient the left ventricular outflow tract at a 30- to 60-degree angle to the ultrasound beam when obtaining all Doppler images. The short axis view was obtained by rotating the probe 90 degrees from the long axis view in a counterclockwise direction. Color flow Doppler cine loops were acquired in long axis and short axis (Figure 2).

**Figure 2**—Two-dimensional echocardiographic images obtained from a koi carp heart as described in Figure 1 demonstrating (A) sagittal view, including the ventricle (V) and bulbus arteriosus (BA), and (B) Doppler outflow tract measurement.
minimum (systole) estimated volume, allowing for the assessment of reliability of the measurements. One observer (radiologist) calculated pulsed-wave Doppler measurements for the left ventricular outflow tract. Measurements for HR and ventricular outflow peak velocity were acquired at the junction of the ventricle and the bulbus arteriosus. Heart rate, ventricular outflow peak velocity, and velocity time interval were all calculated from the pulsed-wave Doppler images.

**Gross anatomy and histologic cardiac assessment**

Two fish were humanely euthanized for gross anatomic and histologic cardiac evaluation and description by a veterinary pathologist. Euthanasia was performed by overdose (500 mg/mL) of tricaine methanesulfonate (Tricaine-S Western Chemical, Inc, Ferndale, WA) buffered 2:1 with sodium bicarbonate. Prior to dissection, fish were observed for absence of respiratory movements for 15 minutes. For histologic evaluation, tissues were fixed in 10% neutral-buffered formalin for 24 hours before routine processing, sectioning at 4 µm, and staining with H&E. Additional special staining procedures performed on archived koi heart tissue blocks included the Verhoeff van Gieson, Masson trichrome, and phosphotungstic acid hematoxylin stains. Prepared slides were observed using a light microscope (Olympus BX41; Olympus Corp) and digital camera (DP71; Olympus Corp). Select sections were scanned using a digital slide scanner (Aperio AT-2; Leica Biosystems).

**Statistical analysis**

For the responses obtained by expert assessment of echocardiograms (EF, SV, and CO), a linear mixed-effect model suitable for the crossover design of the study was fitted. The model included main effects of treatment, order of treatment, veterinarian, image, and subject (fish). For HR and OPRs, a similar model was used for analysis except that it lacked veterinarian and image effects since these objectively measurable responses were assessed just once per fish per measurement occasion. In these models, subject effects were treated as random to account for heterogeneity from subject to subject, and random image effects were included to account for inter-rate correlation when assessing the same image. All other factors in the models were treated as fixed effects. To correct for heteroscedasticity in SV and CO, these variables were analyzed on the natural logarithmic scale. Marginal treatment means and their differences were estimated from the fitted model for each treatment and any other statistically significant main effect (eg, for veterinarian when significant). These means and mean differences are reported on the original scale for all variables.

To assess reproducibility, we calculated Pearson correlations and limits of agreement (LOA) between 2 veterinarians. These statistics were computed separately for each treatment for EF and SV, where the latter variable was log-transformed because it exhibited increasing variance with the mean and nonconstant disagreement between radiologists on the original scale. Sample correlations and LOA were similar across treatments, so these quantities were visualized with a scatter plot and a Bland-Altman plot, respectively, using all data pooled across the 2 treatments.

All statistical analyses were implemented in R Statistical Software (R Core Team, version 4.3.0) using packages lme4, lmerTest, emmeans, and tidyverse. A threshold for statistical significance of 0.05 and a confidence coefficient of 95% for CIs were used throughout the statistical analyses.

**Results**

Both manual restraint and anesthesia were successful methods to perform echocardiography in koi in this study. The majority of fish showed sedation effects and lateral recumbency within 5 minutes of isoeugenol immersion. The total procedure time was similar between groups, with mean total procedure times ± SD of 8.5 ± 3.04 minutes under manual restraint and 7.7 ± 2.78 minutes under anesthesia. Intermittent spontaneous movement occurred in manually restrained koi. No adverse effects from handling or anesthesia were noted following the examinations. Mean ± SD snout-to-vent length was 53.02 ± 3.39 cm.

The results are presented as mean (SE) for EF, SV, HR, CO, and change in OPR (Table 1). A significant difference was found between manual restraint and anesthesia for EF, SV, HR, CO, and change in OPR.

Table 1—Echocardiographic measurement results in 40 clinically healthy adult, unknown sex, privately owned koi carp under manual restraint (n = 40) or isoeugenol at 50 ppm (n = 40) on May 10 and 11 and June 26 and 27, 2021, including ejection fraction, stroke volume, heart rate, cardiac output, and change in opercular rate from start time 0 to 5 minutes into the procedure.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Isoeugenol</th>
<th>Manual restraint</th>
<th>Treatment difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejection fraction (%)</td>
<td>Mean</td>
<td>SE (95% CI of mean)</td>
<td>Mean</td>
</tr>
<tr>
<td>Stroke volume (mL)</td>
<td>0.788</td>
<td>0.0529 (0.683 to 0.893)</td>
<td>1.079</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>78.9</td>
<td>3.64 (71.7 to 86.2)</td>
<td>68.6</td>
</tr>
<tr>
<td>Cardiac output (mL/min)</td>
<td>57.4</td>
<td>4.43 (48.6 to 66.2)</td>
<td>70.4</td>
</tr>
<tr>
<td>Change in opercular rate (beats/min)</td>
<td>-14.25</td>
<td>2.81 (~19.84 to ~8.66)</td>
<td>9.45</td>
</tr>
</tbody>
</table>

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over time (Table 1). Ejection fraction (percentage) was significantly lower in anesthetized (60.7 [1.1]) compared to manually restrained (64.0 [1.1]) koi (P < .01). Stroke volume (milliliters) was significantly lower in anesthetized (0.788 [0.0529]) compared to manually restrained (1.079 [0.724]) koi (P < .01). Heart rate (beats per minute) was significantly higher in anesthetized (78.9 [3.64]) compared to manually restrained (68.6 [3.64]) koi (P < .01). Cardiac output (milliliters per minute) was significantly lower in anesthetized (57.4 [4.43]) compared to manually restrained (70.4 [5.43]) koi (P = .044). Opercular rate significantly decreased compared to baseline (time zero) by 5 minutes into the procedure in anesthetized koi, with a mean change of −14.25 (2.81) breaths per minute (P = 0). A significant effect of observer (P = 0) was found for EF, SV, and resulting CO. To assess reproducibility, correlation (Kendall tau and Pearson rho) and LOA analysis were performed. Weak correlation and poor agreement were found for both EF (Figure 3) and SV (Figure 4).

Gross and histologic evaluation of the heart was performed (Figures 5 and 6) to aid in the determination of probe placement and cardiac assessment. The koi heart is located on the midline caudoventral to the gills within an inelastic pericardial chamber protected by the pectoral girdle and is comprised of 4 successive physical chambers. The membranous sinus venosus is integrated into the transverse septum and receives venous blood via the hepatic
and paired common cardinal veins. The irregularly shaped atrium is limited by a thin wall and contains a loosely organized meshwork of myocardial trabeculae. Blood passes from the atrium into the ventricle via an atrioventricular valve. Consistent with less-active swimmers, the koi ventricle has a rounded apex. Blood exits the ventricle under high pressure through a semilunar ventriculo-bulbar valve into the distensible bulbus arteriosus that evens the pressure of blood as it enters the ventral aorta.

Histologically, the heart is limited by a 1- to 2-cell squamous epithelium overlying a thin vascularized, fibrocollagenous subepicardial layer. Bundles of loosely associated myofibers form variably thick trabeculae that partition large luminal spaces in the thin-walled atrium. In the longitudinally bisected ventricle, the myocardium surrounds a narrow central lumen and is dominated by a thick inner spongy myocardium and thin outer layer of densely compacted myofibers in a variable ratio of 2:1 near the apex and 4:1 near the ventriculo-bulbar valve. The compact layer is composed of bundles of closely apposed parallel myofibers visualized in layers oriented perpendicular to each other. A sharp line of demarcation denotes the compact and spongy cardiac layers. Myofibers in the spongy myocardium appear as a haphazard syncytium of variably oriented branching and anastomosing cords. Individual cardiac myofibers are 5 to 20 μm in diameter with distinct cross-striations and central elongate nuclei with rounded ends and finely stippled chromatin. An inconspicuous, 1-cell-thick endocardium lines the myocardium. Dominated by elastic connective tissue, the bulbus has an outer layer of circularly arranged fiber groups interspersed peripherally with collagenous bundles that form a dense outer tunic. An inner layer of elastic fibers forms villus-like projections directed centrally into the lumen. In contrast, the cardiac valves are composed primarily of loosely organized collagenous fibers.
Discussion

This study describes an echocardiographic examination technique in koi that was safely performed under both manual restraint and anesthesia with isoeugenol. Echocardiographic examinations were all complete within 20 minutes and complete within 10 minutes in most cases. Koi under manual restraint were more prone to brief intermittent bursts of activity, which varied between individual fish. This resulted in more frequent interruptions during imaging; however, it did not significantly affect the overall examination time, which was similar between both groups. Due to the number of fish included in this study and the methodology requiring multiple measurements by 2 different veterinarians, echocardiographic measurements were performed at a later date using the saved cine loops. In a clinical setting, measurements could be performed in real time for an individual patient.

We hypothesized that cardiac parameters, including HR, SV, CO, and EF, would be statistically different between manually restrained and anesthetized koi and expected to see cardiovascular depression in the anesthetized group. Significant differences were found for HR, SV, EF, CO, and change in OPR between manually restrained and anesthetized koi. These changes were expected based on the cardiorespiratory depressant effects of anesthesia with isoeugenol in other fish species. Isoeugenol at 60 ppm was observed to decrease HR, SV, and CO in Chinook salmon. HR was expected to also be lower in anesthetized koi compared to manual restrained koi; however, the opposite effect was observed. Increased HR could be associated with a response to stimulation by circulating catecholamines, associated with stress or anesthesia, or a compensatory autonomic response to maintain adequate systolic pressure and ensure sufficient blood flow despite a reduction in SV. The examination start time and duration varied based on the individual; thus, the HR and echo measurements were not performed at the exact same time point. Due to this timing variation, initial changes in parameters could have been missed. Further changes in cardiac parameters may have been observed if anesthesia and handling durations were extended and measurements were repeated, which could be considered for future studies.

Although statistically significant differences were found between groups, it is unclear if the differences are clinically significant. Heart rate, SV, and CO are reported to be highly variable between species and with changes in activity in fish. A change in CO in fish is most commonly achieved through SV as fish can achieve much greater increases in SV compared to HR. Stroke volume is dependent on ventricular end-diastolic volume, which results in very high EF, up to 80% to 100% in previously studied fish species. Similar to reports in other fish species, EF in koi in the current study varied between 42% to 90%. Cardiopulmonary parameters in previous studies may vary due to demographics (eg, species, age, sex, or body size) or methodology regarding activity level, restraint (eg, conscious vs different sedation or anesthetic protocol and depth), environmental or water quality factors (eg, temperature or oxygen), and/or measurement technique (eg, estimated or calculated values vs direct measurement with echocardiography or surgically implanted ultrasonic flow probe positioned around the ventral aorta), limiting interpretation and comparison between studies.

Assessment of reproducibility is clinically important in echocardiographic studies as the technique lends itself to variability due to intrinsic biologic variation and differences in measurement and interpretation between operators. Based on the statistical correlation and agreement analyses, EF and SV measurement showed low reproducibility. This is most likely due to the large variation observed in ventricle shape between ellipsoid to spherical throughout the cardiac cycle. The Simpson method is preferred for measuring volume when ventricular shape is variable and has been previously utilized for the assessment of cardiovascular parameters in fish. The trabeculations from the spongy myocardium of the ventricle also resulted in difficulty tracing the myocardial borders and likely contributed to interobserver variability. Based on these findings, echocardiography as described in this study may be best utilized for assessing trends over time in a single individual.

An electrocardiogram was not performed in the current study as leads could not be maintained for a successful reading in the manually restrained fish. The lack of a timing lead ECG during the echocardiogram prevents the identification of electrical systole and diastole, and echocardiographic measurements therefore rely solely on 2-D mechanical estimates of timing throughout the cardiac cycle. As such, end diastole and end systole were visually estimated, which may have contributed to variation in this study. A timing lead ECG is recommended as an additional component to add to future echocardiographic studies.

In conclusion, the results of this study support that echocardiography is a noninvasive method of cardiac assessment that is safe and practical to perform under manual restraint or short-duration anesthesia with isoeugenol at 50 ppm in koi. Although significant differences in multiple cardiopulmonary parameters were observed in the anesthetized fish, the differences were subjectively not clinically significant. All koi maintained opercular movement throughout anesthesia and recovered well. Due to the reported low reproducibility, echocardiography in this species may be best suited for the evaluation of trends over time in individual koi. Future studies are indicated to further evaluate echocardiography under different anesthetic protocols and durations, evaluate echocardiography with the addition of ECG, improve
upon the reported techniques, and compare echocardiographic parameters in koi with cardiac disease.

Acknowledgments

The authors would like to dedicate this research in memoriam of Carl Foss, who was a significant positive force in the koi community and without whom this research would not have been possible. The authors extend thanks to both Carl Foss and Vicki Vaughn for volunteering their time and koi to participate in this research. The authors would also like to thank Nia Chau, RVT, for her assistance in monitoring and recording during the examinations.

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