Advancing early detection technologies for dreissenid mussel invasion: comparing detection of *Dreissena polymorpha* in environmental samples with environmental DNA (eDNA) and scent detection canines

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Abstract

Early detection and rapid response provide cornerstones of effective management of biological invasions in freshwater ecosystems. Tools and technology that increase the sensitivity and efficiency of species detection directly benefit such efforts, increasing the importance of robust comparisons of different detection technologies. In this study, we compared the sensitivity of two detection methods targeting invasive zebra mussels (*Dreissena polymorpha*) in lakes of central Texas: environmental DNA (eDNA) analysis and canine scent detection. eDNA analysis has regularly applied to environmental samples as a component of *D. polymorpha* management programs; however, canine scent detection has typically been limited to inspection for adult mussels on watercraft. Our work represents one of the first attempts to detect *D. polymorpha* veligers (i.e., free-swimming larval stages) in environmental samples with canine scent detection and robustly compare canine and eDNA based approaches. The objectives of this project were to: 1) evaluate whether canines can learn to detect *D. polymorpha* veligers in various water sample background; 2) identify canine and eDNA limits of detection; 3) quantify eDNA performance in river systems; 4) assess traditional, canine, and eDNA technologies screening ten unknown lakes for *D. polymorpha*; and 5) compare economic costs of canine and eDNA methods. In order of objective, our major findings include: 1) canines can be trained to detect *D. polymorpha* veligers; 2) eDNA is 1 to 100 fold more sensitive than canine detection; 3) eDNA can detect *D. polymorpha* eDNA material at least 14 river km downstream from an invaded reservoir; 4) canine scent detection and eDNA appear to be more sensitive than microscopy for *D. polymorpha* detection in environmental water samples, but more work is needed to understand the conditions dictating when canine scent detection or eDNA outperforms the other; and 5) canine scent detection is cheaper than eDNA analysis on a per sample basis, but tradeoffs in temporal efficiency and sensitivity combined with study or management goals will likely dictate when each method is preferable. Overall, comparison of early detection methods reveals strengths and weaknesses of each approach and helps develop the most efficient, sensitive, and operable approach (or combination of approaches) for invasive species detection.

Introduction

The first detection of Eurasian zebra mussels (*Dreissena polymorpha*) in North America occurred in the Laurentian Great Lakes basin in 1988 (Hebert et al. 1989). *D. polymorpha* and the closely related *D. bugensis* (quagga mussels) have since spread rapidly across the United States and Canada, invading lentic and lotic inland waters via both natural and human-mediated dispersal (Bossenbroek et al. 2001; Sieracki et al. 2014). The high fecundity of *D. polymorpha* (1 million eggs produced per year per female; Keller et al. 2003) in combination with the production of free-swimming-larvae (i.e., veligers) facilitate dispersal via water currents and as hitchhikers on recreational boats and other equipment (Johnson and Carlton 1996, Ram and McMahon 1996**,** Johnson et al. 2001). The impacts of *D. polymorpha* invasion have been ecologically and economically devastating. Invasive mussels can impose bottom-up disturbances to local food webs by reducing available water nutrients (Nakano and Strayer 2014) and alter food webs via reduction of food availability for native species (Holland 1993, Fahnenstiel et al. 1995, Miehls et al. 2009). Furthermore, their voracious filter feeding lends *D. polymorpha* status as potent ecosystem engineers that can dramatically increase water clarity, and they manipulate physical habitat structure by establishing dense colonies attached to all available hard substrates—even native mussels, crayfish, and other hard-shelled species. In addition, *D. polymorpha* heavily impact anthropogenic activities, as prevention and removal efforts for recreational equipment, power plants, municipal water facilities, dams, and other human structures have generated considerable expense (Aldridge et al. 2006). Overall, *D. polymorpha* create an estimated cost of over a billion dollars annually in the United States alone (Pimentel et al. 2005).

Thus, high incentive exists to promote effective prevention and management efforts for nonnative *D. polymorpha.* Early detection and rapid response represent cornerstones of effective invasive species management programs (Lodge et al. 2016), indicating that improving methods for *D. polymorpha* detection provides a critical research goal. Traditional, visual methods of *D. polymorpha* detection includes identifying adult mussels via SCUBA surveys and video imaging, and cross-polarized light microscopy to detect veligers (Johnson 1995, Alix et al. 2016, Penãrrubia et al. 2016; Figure 1). Unfortunately, these methods can be time consuming, expensive, and labor intensive. Furthermore, identification of veligers requires considerable taxonomic expertise and microscopy skill due to similar physical characteristics between veligers and other macroinvertebrate larvae as well as visual impairment due to sediments (Baldwin et al. 1996). Nevertheless, timely detection of veligers may reveal rapidly closing windows for management action, as *D. polymorpha* only exists as free-swimming larvae for several weeks before they settle and develop into attached mussels. On the other hand, identification of mobile veligers may offer clues that reproductive adults exist somewhere

within the waterbody, potentially signaling a need to increase surveillance efforts. Two recently emerging methods of *D. polymorpha* veliger detection includes canine scent detection and the analysis of environmental DNA (eDNA).

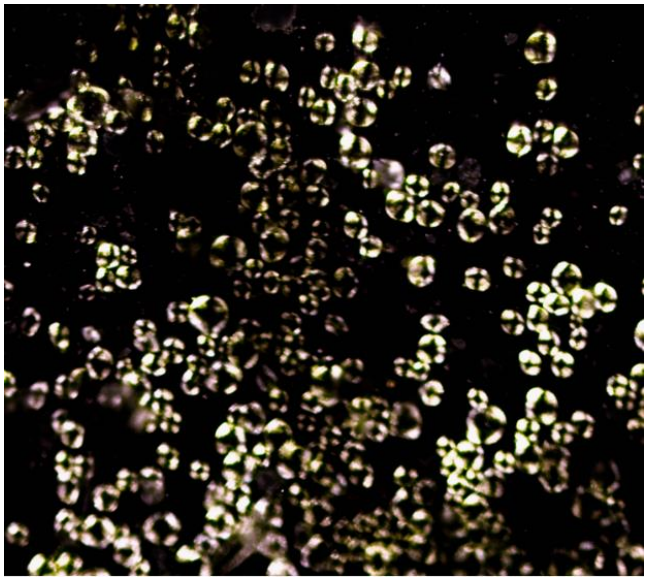

Figure 1. *D. polymorpha* veligers under cross-polarized microscopy, identifiable with the distinct cross shape (4x magnification).

Scent detection canines have been successfully deployed as environmental surveyors for a range of wildlife species. They were capable of yielding more accurate and sensitive results, outperforming standard environmental surveying technologies in detection of different organisms including tortoises and various carnivores (Cablk and Heaton 2006, Long et al. 2007a, Long et al. 2007b, Cablk et al. 2008). Mussel detection canines are currently deployed across the country to survey various watercrafts. Mussel detection canines are often stationed at boat launches to inspect watercraft for the presence of *D. polymorpha* and *D. bugensis* adults prior to entry into an uninvaded waterbody (DeShon et al. 2016). Canines have also been deployed in combination with other mussel surveillance methods such as the use of submerged settling plates (Lucy 2006). More recently, DeShon et al. 2016 have suggested that scent detection canines may provide a sensitive and rapid option for *in situ* detection of veligers in environmental water samples. Although canines have demonstrated success in detecting mussels at all stages of life—including the veliger stage—formal evaluation of detection sensitivity limits are necessary to assess the feasibility of canine detection of *D. polymorpha* veligers.

Analysis of environmental DNA (eDNA), genetic material collected and extracted from bulk environmental samples such as sediment, water, or air (Barnes and Turner 2016), represents another emerging method for rapid and sensitive detection of *D. polymorpha*. Analysis of eDNA

has been applied broadly to the detection of invasive fish (Robson et al. 2016), amphibians (Secondi et al. 2016), and invertebrates, including *D. polymorpha* (Egan et al. 2013, Goldberg et al. 2013, Larson et al. 2017). Demonstrated sensitivity and repeatability have led to the emergence of eDNA analysis as an integral component of many *D. polymorpha* surveillance programs (Amberg et al. 2019, Feist and Lance 2021).

Hence, our goal was to evaluate and validate both canine detection and eDNA analysis for detection of *D. polymorpha*, determining relative strengths and weaknesses of the two detection methods. We evaluated our goal through five objectives that challenge canine and eDNA detection sensitivity limits under laboratory conditions and real-world conditions: 1) Assessing ability to train canines on *D. polymorpha* veliger odor profile in varying water backgrounds; 2) Quantifying sensitivity of eDNA analysis and scent canine detection to veliger presence under simulated laboratory conditions; 3) Quantifying performance of eDNA detecting *D. polymorpha* eDNA in flowing river systems downstream of invaded sites; 4) Screening ten Texas lakes to compare microscopy, canine detection, and eDNA technologies; and 5) Conducting an economic analysis on eDNA and canine detection technologies based on measured accuracy, sensitivity, specificity, and cost per sample. We anticipate that this work will provide the most comprehensive evaluative and validation work for canine detection technology to date and effectively evaluate how eDNA technology compares for *D. polymorpha* detection.

Therefore, we conducted a series of experiments to quantify the sensitivity of eDNA analysis and canine detection of *D. polymorpha*—especially veligers—in environmental samples. This work occurred in Texas, where *D. polymorpha* has been present since at least 2009. Since its first detection in Lake Texoma on the Texas-Oklahoma border, *D. polymorpha* has spread to waters across six river basins in the state (Figure 2). Texas Parks and Wildlife Department (TPWD) classifies *D. polymorpha* invasion status as either “infested” (i.e., the site has a confirmed reproductive population; N = 28), “positive” (i.e., adult mussels or veligers have been detected on more than one occasion, but there is no evidence of reproduction; N = 4 lakes and 7 rivers downstream from infested lakes), or “suspect” (i.e., adult mussels or veligers have been identified once; N = 1).

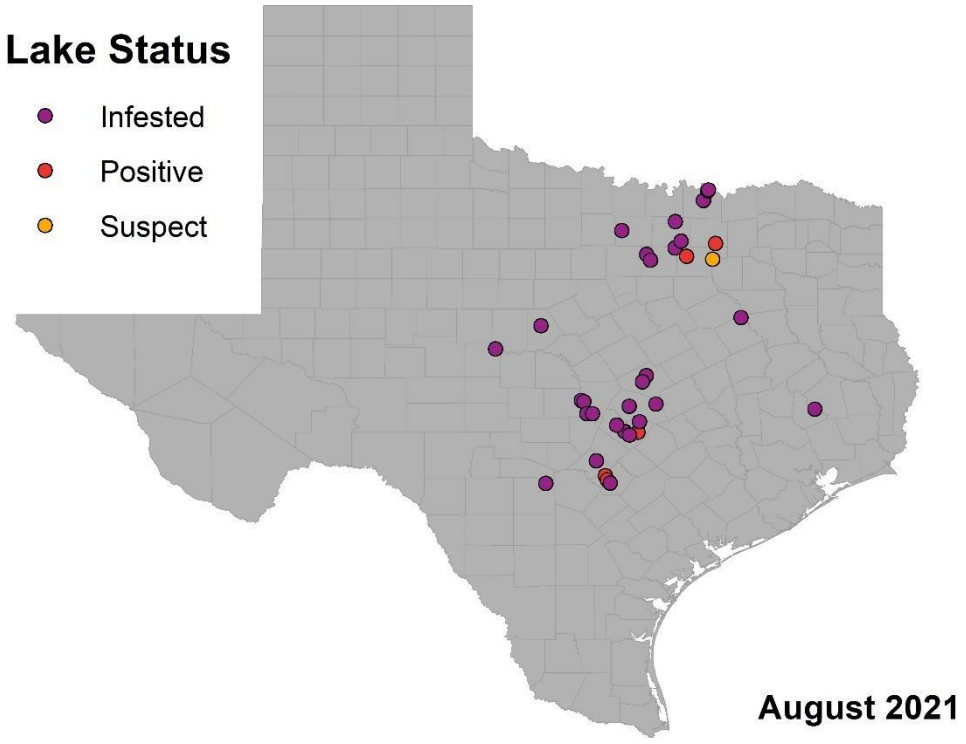

Figure 2. Distribution and status of *D. polymorpha* in Texas. (Map courtesy of TPWD)

Objective 1: Training canines on *D. polymorpha* odor profile.

Methods

Veliger Collection and Preparation. To initially train scent detection canines to identify *D. polymorpha* veligers, we collected and concentrated veligers from Canyon Lake, which is classified as infested by TPWD. We collected veligers using repeated vertical tows of 80-µm plankton nets at the Canyon Lake Marina. Following the method of Johnson et al. (2019), we concentrated collected plankton via sequential filtration through 210-µm and 35-µm sieves and identified and enumerated veligers visually using cross-polarized light microscopy (Figure 3). Depending on the trial (see below), concentrated plankton was resuspended in bottled spring water or filtered lake water from uninvaded lakes. Uninvaded water and plankton communities (i.e., to serve as *D. polymorpha* negative controls) were collected from Calaveras and Braunig Lakes and confirmed to be negative using identical methods.

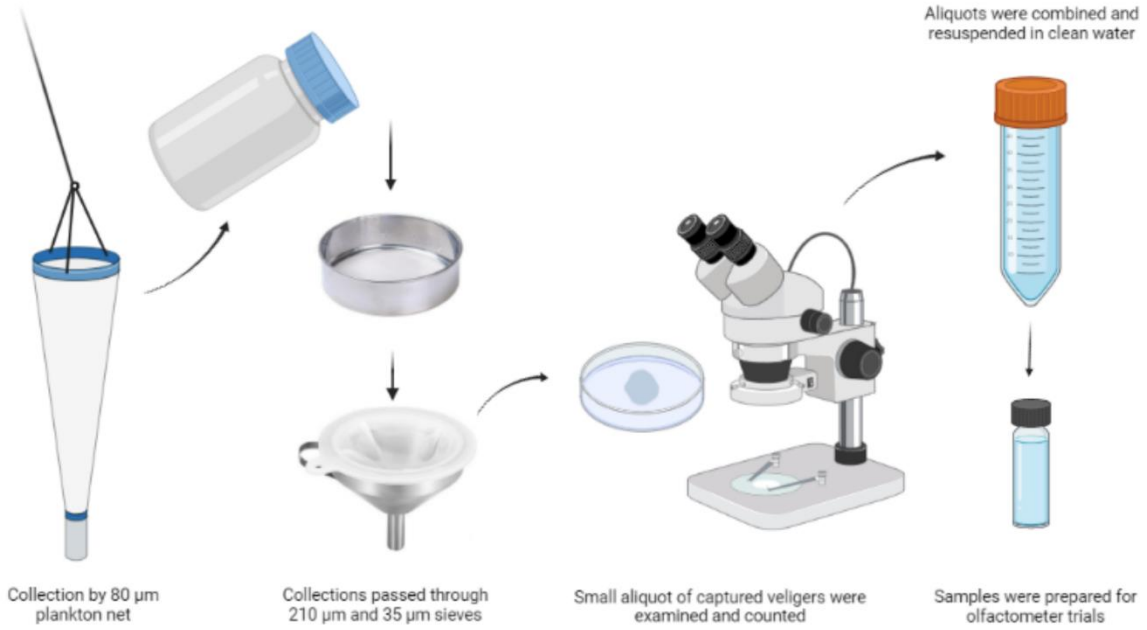

Figure 3. Workflow of collection and sample preparation.

For initial training of canines on *D. polymorpha* odor profile, we collected samples from Canyon Lakes 12-14 September 2021. Positive sample concentration ranged 605-900 veligers/mL resuspended in pure spring water and were stored in 50-mL conical tubes at 4 °C for at least 12 hours to ensure equilibration of the sample with the biological material.

Participants. We measured the performance of six canines employed by Mussel Dogs (https://www.musseldogs.info/; Figure 4), and all trials took place at Chiron K-9 in San Antonio, TX (https://chiron-k9.com/). All canines were reinforced with a preferred toy reinforcer.

Olfactometry and Training Equipment. Canines were trained using an automated olfactometer controlled 3-Alternative Forced Choice test (3AFC) procedure (Aviles-Rosa et al., 2021). A detailed description of how the olfactometer functions and the validation of its odor presentation has been described elsewhere (Aviles-Rosa et al., 2021; Gallegos et al., 2022). Briefly, the olfactometer has the capacity to hold six sealed headspace vials containing a solid or liquid odorant source. Under computer-controlled activation, regulated airflow can be passed through a specified headspace vial, pushing odorant through a mixing manifold with clean air and delivered to a stainless-steel port where the canine can sample the odorant. Infrared beams then measure canines' time sampling the odors in the ports. Canines were trained to hold their nose in the port containing the target for a specified nose "hold" time and not hold their nose in the incorrect port for the same length of time. The nose hold time was modified for each canine based on their proclivity to maintain a nose hold duration and

Figure 4. Canine collaborators. Top row, left to right: Captain (Male, 8 yo); Dory (Female, 3 yo); Edna (Female, 2 yo). Bottom row, left to right: Gilligan (Male, 4 yo); Marlin (Male, 3 yo); Moomba (Female, 3 yo). All canines were spayed or neutered.

previous training. Captain, Gilligan, and Marlin were trained with a 1.5 second hold time and Dory, Edna, and Moomba were trained with a 3 second hold time. Captain, Gilligan, and Marlin were previously trained to "sit" as an alert response whereas Dory, Edna and Moomba were previously trained to "focus" at the odor source. This difference in prior training was related to differences in proclivity to maintain duration of a nose hold and ultimately the length of the nose hold selected for a response.

Correct responses or "alerts" to the port (nose hold >criterion) containing the target odor were marked with a "chirp" signaling a handler to deliver a reward. If the canine made a false alert (held their nose longer than the hold criterion in the incorrect port), a "buzzer" sounded terminating the trial. During initial stages of training, the program was set to "wait for a correct response" ignoring incorrect responses until the canine made a correct response. However, only the first response (i.e., the incorrect response) was scored. This was employed to facilitate initial training allowing canines to correct initial mistakes, but this setting was not used for formal data collection. If the canine sampled all three ports and did not alert within the hold criterion, an "all clear" response was scored. After each trial, the olfactometer conducts a 25 s odor purge and pseudo-randomizes the location for the next port (randomization was balanced across ports such that each port contained the target an approximately equivalent number of

times). All training and testing occurred double-blind, as handlers and experimenters did not know the correct location of the target odor, as this was entirely under computer control.

Detection Training. Canines were initially trained to the odor of *D. polymorpha* veligers minimizing as many background odors as possible. A concentrated sample (5 mL of a ~900 veligers/mL sample was prepared as described above) was prepared using 5 mL of spring water as the diluent and served as the target odor. Clean spring water (5 mL; the same diluent as for the target) served as the distractors/alternative odor. Thus, the aim of this test was to evaluate whether canines could discriminate between spring water containing filtered veligers from clean spring water. Canines were trained until exceeding 8 or more correct responses in a 10-trial session (binomial test $p < 0.01$). Canines were tested in a 10-trial session to confirm detection proficiency with new samples and to qualify for advancing to the next detection phase.

Detection Certification. Once canines exceeded 8 or more correct responses in a 10-trial session in Detection Training, canines were trained by adding Braunig lake water to all samples to make a 50% spring water and 50% filtered Braunig lake water solutions. After canines exceeded greater than 8 of 10 correct responses, canines were tested in a 10-trial certification test in which a novel concentrated sample (~825 veligers/mL) was prepared collected from Canyon Lake and the diluent for all samples was filtered Braunig Lake water. The goal of this test was to confirm veliger detection in a complex lake water background from control samples of otherwise identical lake water.

Control Test. Immediately following detection training, canines were given a 10-trial control session. In this session, all samples were replaced with only spring water (no target odor sample), and canines were trained identically. If canines had learned to use an unintentional cue provided by the olfactometer that was unbeknownst to the experimenters, it would be expected that canines would continue to perform above chance (i.e., 33% correct, corresponding to 1 of 3 ports presenting the positive odor in each trial) in the absence of the target odorant. In contrast, if the canines were detecting only the veliger sample, removal of that sample would cause canine performance to drop to chance levels.

Plankton Control Test. One limitation to the previous tests is that during the veliger concentration step, additional unknown plankton may be concentrated in addition to the target veligers. Thus, it would be possible that canines maybe detecting not just veligers, but rather increased overall plankton components in the target samples introduced by the concentration step. To confirm that *D. polymorpha* veliger presences is critical to canine responding, we conducted a test in which plankton was identically concentrated from several lakes and

reconstituted into different filtered water sources and evaluated whether canines maintained responding associated with only with veliger positive plankton samples. Table 1 provides a description of the different samples. During these testing sessions three targets and two negatives were used. On each individual trial only one target appeared in one of the ports and the other two ports contained the same or different negative samples.

Table 1. Different samples created for the Plankton Control Test.

Concentrated Plankton Source	*D. polymorpha* Status	Diluent filtered water source	Sample Status
Canyon Lake	+	Canyon	Target
Canyon Lake	+	Braunig	Target
Placid Lake	+	Placid	Target
Calavaras	-	Calavaras	Negative
Braunig	-	Braunig	Negative

Biosafety Measures. Throughout research activities related to Objective 1 and all other objectives in this report, we applied strict biosafety measures to prevent inadvertent spread of *D. polymorpha* via contaminated equipment. Specifically, after each use, plankton nets soaked in vinegar for 1 hour, followed by a 10% bleach soak for 10 minutes, then rinsed and dried overnight. Other equipment (i.e., bottles, petri dishes, forceps) was decontaminated by soaking in 10% bleach for 10 minutes, rinsed, and dried completely between uses. Water was collected under the auspices of Texas Parks and Wildlife Exotic Species Research Permit No. RES 08 21-157. In addition, all vertebrate animal work was reviewed and approved by the Texas Tech Institutional Animal Care and Use Committee (AUP # 21013-03).

Statistical Analysis. Mean accuracy (number correct response/total trials) was calculated for the detection training, detection certification and control tests. An individual performance was considered above chance for a 10-trial session if performance was 7 or more correct (binomial test p=0.02). Overall group performance was compared to theoretical chance (1 out of 3, p=.33) using a one-sample T-test. To evaluate the results of the Plankton test, the frequency of alerts to each odor type was calculated by scoring the number of trials a canine alerted to an odor by the number of trials the odor appeared. A logistic mixed effect model was used to predict the probability of an alert by each odor type with a random intercept fit for each canine. Tukey adjusted post hoc tests were conducted to compare between each odor tested. Analyses were conducted in R using *lme4*, *lmerTest* and *emmeans* packages (Bates et al., 2013, p. 4; Kuznetsova et al., 2014; Lenth, 2016).

Results and Discussion

All canines exceeded the individual significance criterion (>7 out of 10) for the Detection Training and Detection Certification while no canine met this criterion for the control test (Figure 5). At the group level, canines exceeded performance expected by chance on the Detection Training ($p < 0.001$) and Detection Certification ($p < 0.001$) but not on the control tests ($p = 0.37$), indicating canines were correctly indicating the *D. Polymorpha* samples and were not responding to unintentional non-odor related cues.

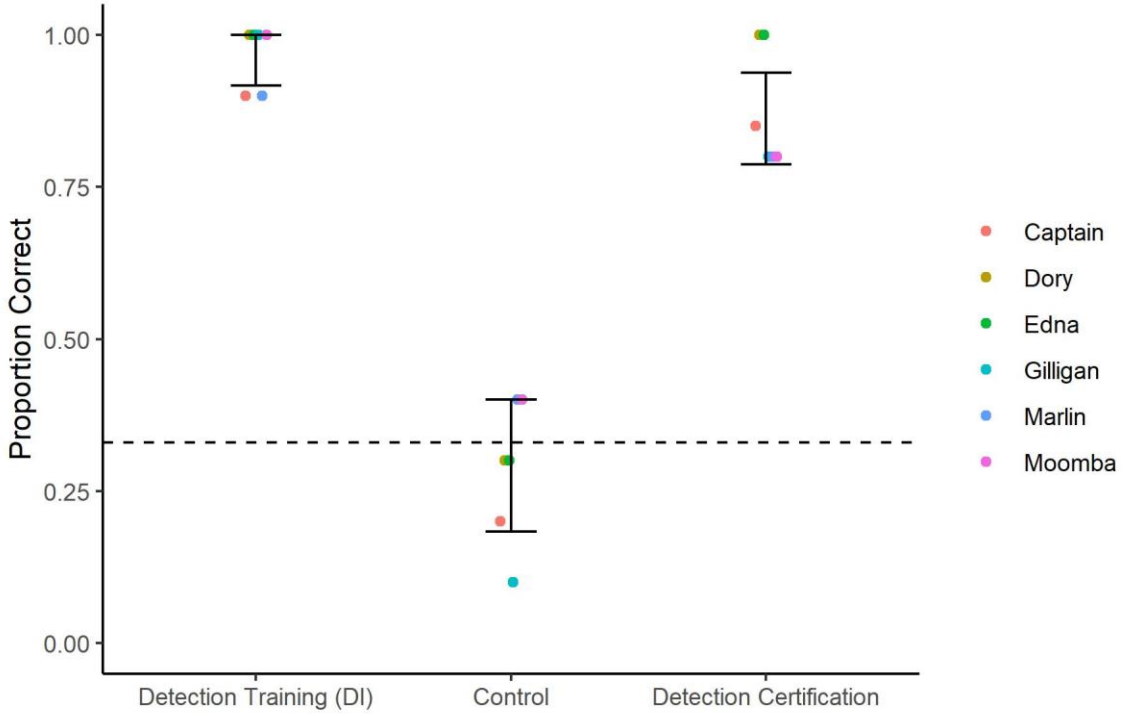

Figure 5. Veliger Detection. Shows each canines' detection accuracy (chance=0.33; dashed line) on the detection training (veliger detection in DI water from DI distractors), the control test (all DI water) and the detection certification (veligers re-suspended in clean lake water from clean lake water). Error bars show the bootstrap estimated 95% confidence intervals.

Canines showed substantially greater responding to all samples with the plankton component from veliger positive lakes compared to the samples with concentrated plankton from veliger negative lakes (all $p<0.001$; Figure 6). Interestingly, canines did show lower alert rates for veliger positive plankton from Canyon Lake re-constituted into Canyon Lake compared to the veliger positive plankton reconstituted with clean lake water from Braunig ($z = 4.14$, $p < 0.001$).

This may reflect that the canines had prior experience with veliger positive plankton being reconstituted with Braunig water, and this was the first use of Canyon Lake water as a diluent. However, no decrement was observed for Placid samples which was an entirely novel source of *D. polymorpha* and diluent source.

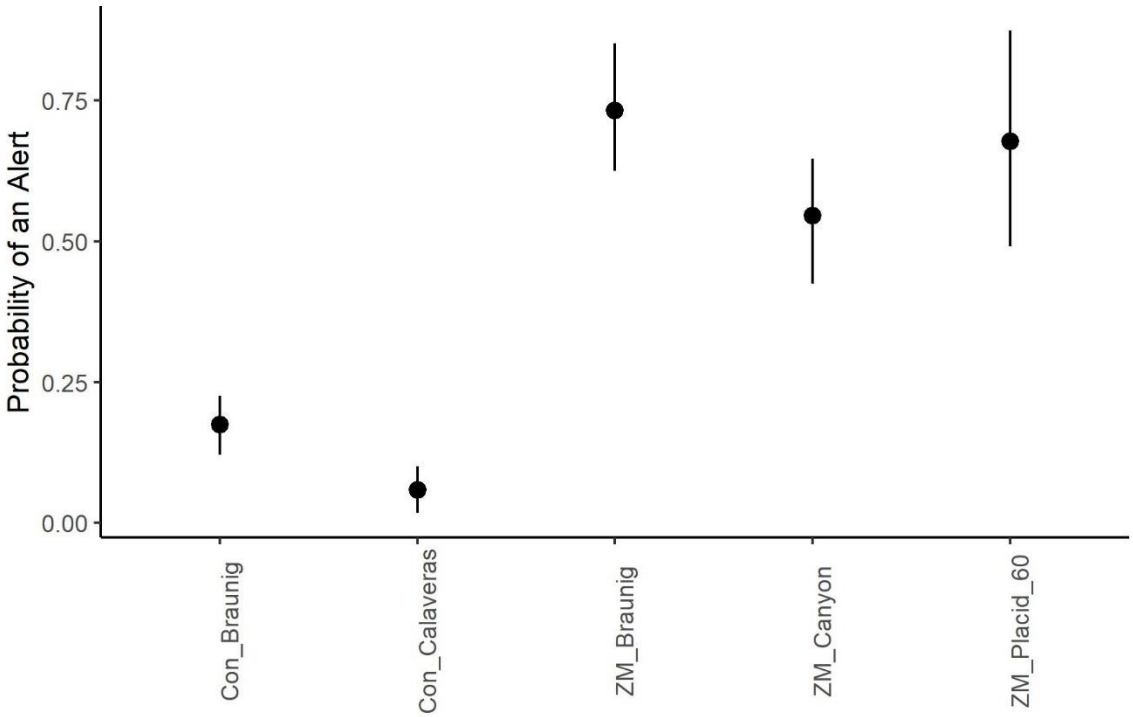

Figure 6. Responses to each odor during the Plankton Control Test. Points show the mean and error bars show the bootstrap estimated 95% confidence intervals.

Together, these results demonstrate that canines can successfully learn to discriminate (1) clean spring water samples with concentrated veligers from otherwise identical clean spring water samples (2) lake water samples with concentrated veligers from filtered lake water samples and (3) lake water samples with concentrated veligers from lake water with concentrated veliger-free plankton. These results therefore strongly indicate canines can detect *D. polymorpha* veliger samples in lake water when concentrated 825 veliger/mL or more.

There remains a question as to what concentration of veligers is required for canines to respond appropriately. At a concentration of 825 veligers or more, substantial concentration of a sample is required. However, it would be useful to know the minimum concentration of the veligers required for an accurate detection for both canines and eDNA.

Objective 2: Quantify limits of detection of canine olfaction and eDNA analysis under simulated laboratory conditions.

Given that Objective 1 showed positive results that canines can detect lake water samples with concentrated levels of veligers and discriminate these from highly similar and overlapping samples without veligers, it is worth investigating the minimum concentration that was sufficient for canine detection. The aim of Objective 2 was to establish the sensitivity curve for veliger detection for each canine and for eDNA analysis.

Methods

Veliger Collection and Preparation.

Following identical methods to those used for Objective 1, we prepared a concentrated sample of 837 veligers/mL resuspended in filtered Lake Calaveras water. From the initial concentrated sample, a serial dilution in half-log steps was made in quadruplicate (5mL aliquots in amber vials). Three sets of serial dilutions were used for canine evaluation in the olfactometer (one set for each olfactometer). The fourth series was used only for eDNA analysis.

Canine Olfactory Detection Sensitivity.

Canines were tested using the same olfactometer system described in Objective 1. Detection sensitivity limits were testing using a serial dilution series (half-log dilutions) and canines were tested under a 2-down 1-up descending staircase procedure (DeChant & Hall, 2021; Leek, 2001). In this procedure, if canines make two consecutive correct responses, the concentration of the odorant is decreased in a half-log step. If an incorrect response is made, the concentration is increased by a half-log step. Testing continues until eight reversals in the direction of concentration change (up or down) occurs or 40 trials occurs. Due to olfactometer limitations with the capacity for only six channels, only 5 dilutions were trained at a time, with the final channel being reserved for a control. If the canine successfully detected all 5 dilutions, the canine would then re-start threshold assessment with a new range of dilutions starting with the lowest concentration successfully detected. Testing continued until all 8 reversals were completed, or the canine reached 40 trials without either meeting all reversals or completing all dilutions. During the lowest 6 dilution steps, the olfactometer was changed to contain only three dilution steps and three control samples. This was done to reduce the over-use of the odor from control vials and headspace of control lake water from dissipating across testing when canines required numerous trials to detect the sample at lower concentrations. Each canine completed two complete threshold assessments (8 reversals). After every canine completed their first threshold assessment, an

identical control test to the first was conducted to verify no unintentional cues were being leveraged by the canines.

eDNA Technology Sensitivity. To assess eDNA sensitivity to veligers, the fourth series of serial dilutions, were vacuum filtered using 1-µm polycarbonate 47-mm membrane disk filters (Whatman). We extracted eDNA from filters using a CTAB-Chloroform extraction (Turner et al. 2014), then applied a species-specific quantitative PCR assay targeting *D. polymorpha* CytB gene (Gingera et al. 2017) to quantify *D. polymorpha* eDNA. Each 20-uL qPCR reaction included 1 uM forward and reverse primers, 0.6 uM hydrolysis probe, 1x PerfeCTa qPCR ToughMix (Quantabio, Beverly, MA, USA), and 4 uL template DNA. To enable absolute quantification, we prepared a four-point serial dilution of *D. polymorpha* tissue-derived DNA quantified using a Qubit fluorometer and broad range dsDNA sensitivity kit and (Life Technologies, Grand Island, NY, USA). All samples were run in triplicate, and quantified values were averaged. Results were summarized as limit of detection (i.e., at least 1 of 3 technical replicates positive) and limit of quantification (i.e., 3 of 3 technical replicates positive).

Statistical Analysis. To calculate canine thresholds, the performance of each canine at each dilution was combined across the two threshold assessments. A probit psychophysical function was fit for each canine using the *quickpsy* package where the guess rate was set 0.33 (Linares & López-Moliner, 2016). The 75% detection threshold was estimated for each canine and 95% confidence intervals extracted.

To assess whether canines were using only the target odor for detection performance, data from the control test were analyzed using a one-sample t-test. The mean performance of canines was compared to the theoretical level of chance as 33%. If canines showed a performance significantly exceeding chance in the control test (all vials contained spring water), this would suggest canines were utilizing an uncontrolled cue. The program was otherwise run using identical settings as the threshold assessment.

Results and Discussion

Canine Olfactory Detection Sensitivity. Canine detection declined as the concentration of the concentrated veliger sample decreased toward each individual's threshold detection limits (Figure 7).

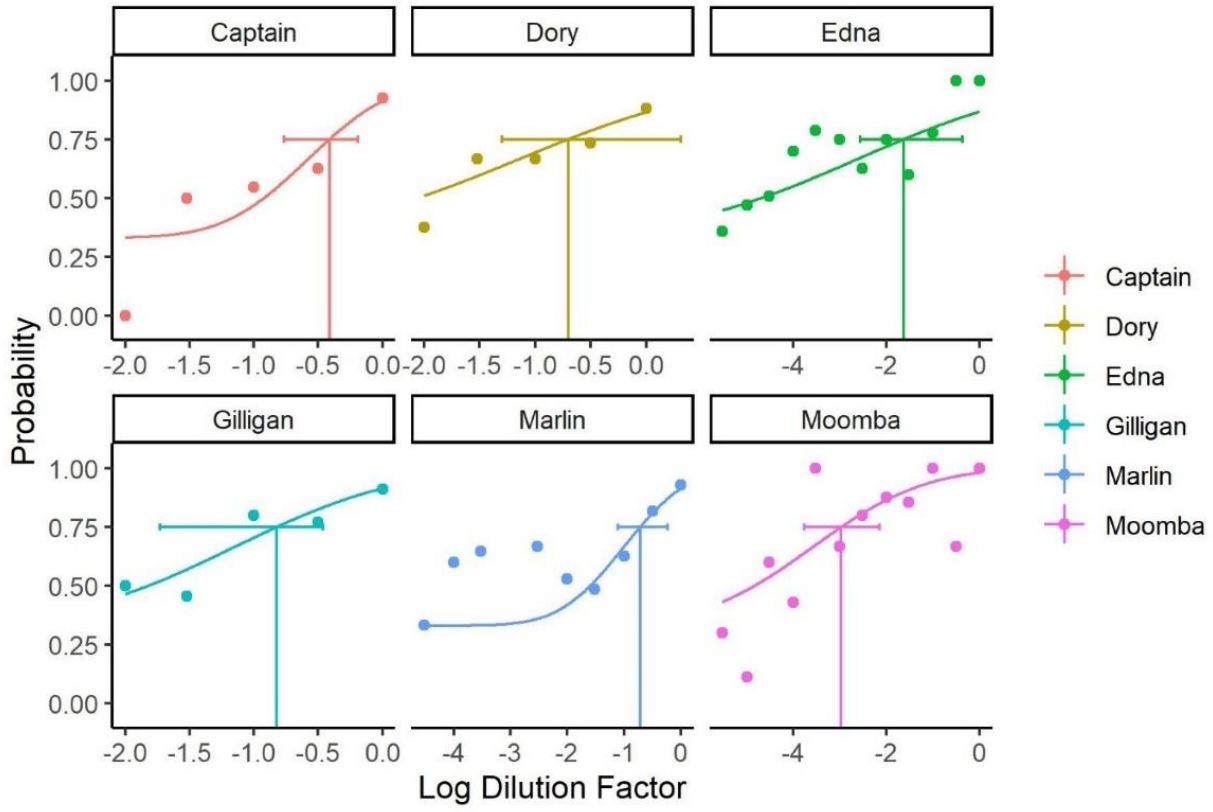

Figure 7. Performance of each canine across dilution levels of the concentrated veliger sample. Error bar shows the confidence interval for the 75% detection threshold.

Four of six canines showed detection limits within a 15-38% dilution of the concentrated veliger sample whereas two other canines showed substantially lower detection limits ranging from 2% to 0.1% dilution of the veliger sample (Table 2). These results suggest a detection limit range of 0.825-313 veliger/mL.

Overall accuracy on the second control test conducted during sensitivity testing yielded an accuracy rate of 40% compared to the likelihood of randomly selecting the correct port from an olfactometer trial or “chance detection” of 33%. A binomial test indicated that this is not a significant deviation from that expected by chance (p=0.27), indicating canines were not leveraging unintentional cues.

Table 2. Estimated 75% detection threshold and 95% confidence interval. Expressed in terms of the dilution factor of the concentrated veliger sample (3.42 mL of a counted 825 veliger/mL sample)

Canine	Threshold Estimate	95% CI Lower Limit	95% CI Upper Limit
Captain	0.386398	0.171399	0.645822
Dory	0.197756	0.050426	2.044848
Edna	0.02348	0.002702	0.420958
Gilligan	0.14969	0.018631	0.344982
Marlin	0.190362	0.077747	0.590732
Moomba	0.001065	0.000173	0.007

eDNA Technology: Sensitivity Determination. All qPCR non-template controls indicated an absence of *D. polymorpha* eDNA as expected. The standard curve efficiency of 92%, and R^2 of 0.999 indicate a robust ability of the assay to accurately quantify *D. polymorpha* eDNA within samples. Rate of positive within sample replicates ranged from 1 out of 3 replicates to 3 out of 3 replicates. Based on amplification, the limit of detection (LOD) was 0.026 veligers/mL that amplified 16.67% of the time. Based on the standard curve, the limit of quantity (LOQ) was 26.45 veligers/mL that was quantified 100% of the time. One of six technical replicates of extraction negative controls had a Ct value of 39.14, corresponding to an average concentration of 0.000006 ng/µL DNA, which is below the limit of detection of the eDNA assay, but still indicative of minor levels of contamination occurring during eDNA extraction of samples for Objective 1. Since the suspected contamination level was considerably lower than experimental DNA detections in the experiment, it is unlikely that this contamination influenced inferences of this trial.

Together, these results indicate that canines show important individual variability between canines in terms of detection sensitivity. The least sensitive canines showed decreases in performance at 313 veligers per mL whereas another canine was showing detection closer to 1 veliger/mL. Interestingly, this suggests that an odor signature of D. polymorpha veliger presence is still available in a substantially diluted sample that may only reflect an anticipated concentration of ~1 veliger/mL. It is important to note, however, that this was only one canine, and that most canines needed tens to hundreds of veligers for adequate detection.

The eDNA limits of quantification were similar to canines with all replicates quantifying at a concentration of 26 veligers/mL. This exceeds the 75% detection limit for all but two canines and exceeds the 100% detection range for all canines. In terms of limits of detection, in which at least one replicate detected *D. polymorpha*, eDNA could detect 0.026 veligers/mL, indicating

a 30- and 12,000-fold improvement over the most and least sensitive canines, respectively. Together, these results suggest that eDNA is a more sensitive tool, with limits of quantification indicating a 1- to 100-fold greater sensitivity and limits of detection indicating a 30- to 12,000-fold greater sensitivity compared to the most and least sensitive canine.

Notably, canines were not selected for the project for specific sensitivity to *D. polymorpha* veligers. Canines were instead previously proficient and professionally deployed for detection of adult *D. polymorpha*. Thus, as a potential deployable tool used to monitor bodies of water, practitioners could use sensitivity testing to identify canines with the most sensitive detection limits.

Additionally, canines within the study period needed training to a novel olfactometer system and to *D. polymorpha* veligers. These two training steps were completed over two weeks based on study timelines and *D. polymorpha* reproductive seasons. Threshold and detection sensitivity limits are related and known to change with increased training and experience (e.g. Hall et al., 2016; Yee & Wysocki, 2001). Thus, these sensitivity limits do not necessarily reflect the maximal achievable sensitivity limits, but rather the sensitivity limits obtained following initial training. Canines could therefore show enhanced detection or more considerable similarity across canines with extended periods of training (months or more) that were not feasible within the scope of the project.

Some of the variability between canines could also potentially be related to differences in canines' proclivity for the nose hold alert. Gilligan, Captain, and Marlin had reduced criterion for a nose hold due to previous training for a "sit" alert response. This reduced nose hold time may be related to some of the variability in performance at lower concentrations because these canines did not have as much "nose in port" time sampling the odor. Some canines also have a response of sitting instead of leaving their nose in the port. The degree to which this difference impacted results is not clear because the canines' previous training was not a controllable/manipulable variable. Nonetheless, previous research has suggested that the type of response a canine makes can have performance effects (Essler et al., 2020). Thus, the type of response a canine is trained to make, and its impact on detection sensitivity would be a useful evaluation in future research.

Objective 3: Determine eDNA downstream detection capability to *D. polymorpha* veligers of known mussel beds in rivers.

With the detection limit range determined for canines and eDNA under simulated laboratory conditions, it was appropriate to turn to real-world conditions to evaluate and compare canine and eDNA capabilities. The goal of Objective 3 was to assess canines' ability to detect veligers in unconcentrated samples and eDNA analysis in downstream detection of known mussel beds sites.

Methods

Canine Detection of Veligers from Non-Concentrated Samples. Objective 2 demonstrated that canines can have notable sensitivity for the presence of veligers in a water sample, but most canines' sensitivity was not sufficient to detect veligers present in non-concentrated samples (i.e., ~150 veligers per mL). This suggests that canines may need samples to be collected from plankton nets repeatedly and concentrated. This, however, was not possible in many downstream sampling sites. We therefore conducted an assessment to evaluate whether canines were able to sufficiently detect samples from a single plankton net pull from a veliger positive lake from that of a veliger negative lake to evaluate whether a further concentration step in necessary for adequate canine detection.

Trials in which a sample of water from a single net pull from Canyon Lake were collected and distributed into 5mL aliquots for the olfactometer. Additionally, a concentrated sample (~825 veligers per mL) was prepared from Canyon Lake and re-constituted with Calavaras water (see Objective 2) and then diluted to 10% (~82 veligers per mL), which nearly all canines showed proficiency with during the dilution series. This sample served as a positive control and to motivate canines on the task if they struggled to detect the single net pull samples. Distractors used were filtered Calavaras water in addition to water collected from a single net pull from Calavaras Lake and Braunig Lake. Canines were trained in six sessions of 10-20 trials per session where 80% of the trials contained a target odor (equal probability of the 10% dilution of the concentrated veliger sample from Canyon Lake or the single net pull from Canyon Lake). The remaining 20% of trials contained all distractor samples in which canines were expected to provide an "all clear" response.

eDNA Sample Collection and Preparation. The two systems chosen for this objective were the Guadalupe River, which empties Canyon Lake, and the Lampasas River downstream from Stillhouse Hollow Lake (Figure 7). Both lakes are classified as infested by Texas Parks and Wildlife. Up to four sites were chosen along the rivers, with one upstream site chosen as close

to the dam as possible as a positive control (i.e., “Site 0”). We sampled eDNA at Site 0 and sites moving downstream from each reservoir based on accessibility via bridges, highways, and roads crossing over the rivers, up to a maximum of 14 river km downstream. Water sampling took place between September 22-28th, 2021. Water samples were taken from the furthest downstream site to the most upstream site to prevent potential contamination. Three 500 mL bottles of surface water were collected from each site. All bottles were previously soaked in 10% bleach water for 10 minutes to prevent contamination. For each sampling day, an additional 500mL bottle of distilled water was placed in the cooler with the samples to serve as a negative control (“field blank”). Samples were collected with a clean pair of nitrile gloves at each site to avoid contamination between sites.

Within four hours of collection, samples and field controls were vacuum filtered through Whatman Nuclepore Track-Etch Membrane 1µm pore filters. Used supplies (i.e., filter apparatus, forceps) were soaked in 10% bleach prior and after use. Each sample was removed from the cooler and the outside of the bottle was rinsed with tap water to further remove contaminants. Each sample was filtered in its entirety or for five minutes, whichever came first. Each 1µm pore filter from the same sample was placed in the same 1.5mL plastic tube and stored in the freezer until DNA extraction. DNA extraction and quantification with qPCR proceeded as in Objective 2, except in qPCR, six technical replicates were run for each field sample.

Results and Discussion

Canine Detection of Veligers from Non-Concentrated Samples.

Canines showed proficient detection of the 10% dilution of the concentrated veliger samples, however canines largely failed to detect the netted samples across the six sessions, with the 95% confidence intervals largely overlapping chance (p=0.33; Figure 8). These results indicate that while the canines were proficient when samples are concentrated, a single net pull was insufficient, indicating that in areas downstream where repeated net pulls may not be possible, canine evaluation may not be a feasible detection tool.

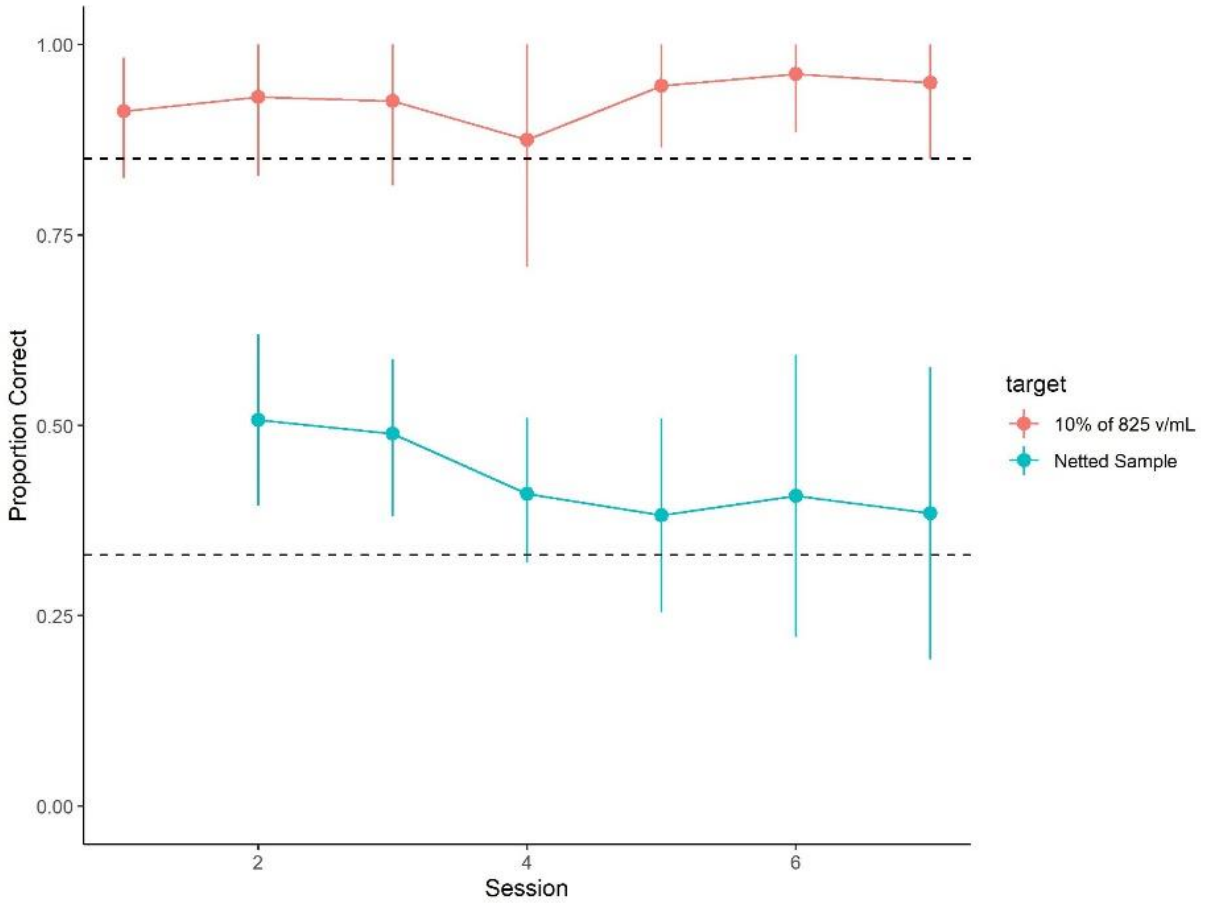

Figure 8. Canine detection of Netted Samples. Points show mean and error bars show 95% confidence intervals.

eDNA Technology: Downstream Detection. Extraction and qPCR negative controls all indicated an absence of *D. polymorpha* DNA, as expected. Standard curve efficiency ranged from 85.9% to 91% with R^2 between 0.988 and 0.999, demonstrating a robust ability to quantify *D. polymorpha* eDNA. Across the 9 sites sampled in the Guadalupe River (downstream of Canyon Lake) and the Lampasas River (downstream of Stillhouse Hollow Lake), *D. polymorpha* eDNA was detected (Figure 9). Rate of positive detection among water samples ranged from 1 out of 3 samples to 3 out of 3 samples. Within water samples, rate of positive detection among replicates ranged from 1 out of 6 replicates to 6 out of 6 replicates. Sample detections that had at least one positive reaction included non-detect reactions assigned as a zero concentration when calculating the average quantification. Non-detection reactions are expected low-level DNA analysis and are advised to be included by Ellison et al. 2006. Once quantity averages were calculated, a pattern suggested *D. polymorpha* eDNA decreased while moving downstream.

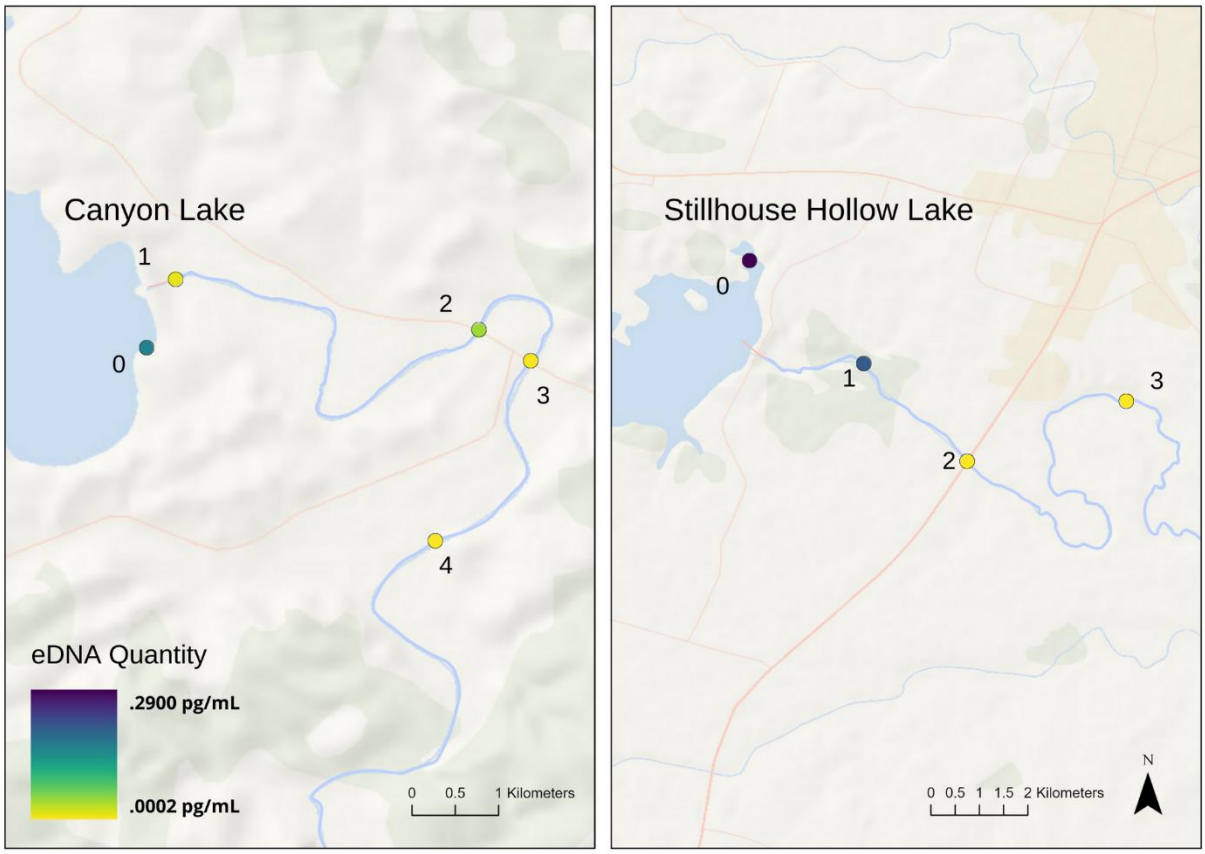

Figure 9. (Left) Map of *D. polymorpha* eDNA detection in the Guadalupe River with eDNA quantities at each downstream. (Right) Map of *D. polymorpha* eDNA detection in the Lampasas River with eDNA quantities at each downstream. *D. polymorpha* eDNA was detected in at least one sample at every site, with quantities generally decreasing with downstream distance at both sites.

The results demonstrate eDNA methods can be used to detect *D. polymorpha* genetic material as far as 14 river km downstream from an infested lake (Figure 9). At Stillhouse Hollow Lake Site 1, the quantity of eDNA was only slightly less than Site 0 (positive site), despite having an approximate distance of 4 km between the two sites. A combination of possibilities could explain the eDNA quantity similarity including the slow rate of eDNA degradation, the resuspension of eDNA in benthic substrates, or the presence of an isolated *D. polymorpha* bed between the two sites (Samson and Sassoubre 2017, Shogren et al. 2017). Further downstream, the drastic difference in quantities between Site 1 and Site 2 at Stillhouse Hollow Lake is puzzling. Despite approximately 2 river km between sites, the quantity of eDNA drops at least thousandfold. An explanation of this could be a higher degradation rate between the two sites because of higher water temperature or microbial abundance at Site 0 (Tsuji et al. 2017, Jo et al. 2019).

Quantity of eDNA detected downstream from Canyon Lake declined rapidly between Site 0 and Site 1, which were less than half a river km apart (Figure 9). Possible explanations could be the retainment of eDNA on the lake shore or the high degradation rate of eDNA due to high water turbulence from recreational sports, resulting in lower detection rates at Site 0 (Laporte et al. 2020, Sakata et al. 2020). Unexpectedly, the eDNA quantity range at Site 2 of Canyon Lake was higher than the quantity range of Site 1, despite having a separation of approximately 5 river km. This could indicate high water flow at Site 1, preventing the eDNA from settling to the benthic floor, or potentially the presence of a small, isolated mussel bed between the two sites, increasing the concentration of eDNA at Site 2. Further research is needed to pinpoint the reason for these unexpected results at both Canyon Lake and Stillhouse Hollow Lake.

Downstream transport of eDNA is an understudied area of eDNA ecology, since eDNA transport is difficult to characterize in flowing river (i.e., "lotic") systems. Demonstrated in multiple studies, eDNA interacts with its surrounding environment such as local biota and inorganic substrates rather than acting like a conservative tracer (Jerde et al. 2016, Shogren et al. 2016, Shogren et al. 2017). Our work provides further evidence of this. Further research should cover a wider range of sites at different lakes and rivers to explore whether the pattern seen in our experiment is the general outcome of *D. polymorpha* eDNA dispersal in lotic systems. Expanding our knowledge of the interaction of eDNA with lotic systems (i.e., rate of degradation, PCR inhibitors, interaction with particles) could also be beneficial. By understanding the dynamic of *D. polymorpha* eDNA in lotic systems, management efforts can be implemented utilizing eDNA methods to better understand *D. polymorpha* dispersal.

Objective 4: Screen ten Texas lakes to compare microscopy, canine detection, and eDNA technologies.

The results from Objective 3 indicate it is not possible for canines to detect veligers in a downstream site whereas eDNA detection is more likely. Given the data, it was logical to compare the abilities of canines and eDNA analysis as well as the traditional method of microscopy in a more traditional location: lakes. The purpose of Objective 4 was to screen ten different lakes of varying infestation status with canines, eDNA analysis, and microscopy and compare their results to determine the outcome of each method and consistency across methods.

Methods

Sample Collection and Preparation. To assess the canines' sensitivity to Texas lakes, a total of ten novel sites were chosen for this objective as shown in Figure 11. These lakes did not include Canyon Lake, Lake Calaveras, or Braunig Lake due to their previous use as training stimuli. Collections were taken September 28th- October 7th, 2021, via plankton net on accessible boat ramps or along the lake shore as described in Objective 1.

Three liters of netted water and three 500 mL of scooped water was collected per site. The 3 liters of netted water were further prepared for canine detection as described in Objective 1 (Figure 3), while the three 500 mL were used for eDNA analysis. For each sampling day, an additional 500mL bottle of distilled water was placed in the cooler with the samples to serve as a negative control (field blank). In this objective, post-filtration veliger counts were not necessary, however cross polarized light microscopy was performed after the canine results were collected to compare a traditional method (microscopy) to canine and eDNA detection results.

Microscopy: Site Detection. An aliquot of 1mL concentrated netted water from each of the lakes was inspected using microscopy with Fien Optic FZ6T-TS Microscope at 4x magnification. The presence or absence of veligers from each lake was recorded as "Detected" or "Not Detected" (Table 4).

Canine Olfactory Detection: Site Detection. Lakes were screened in brief 10-trial sessions. The session was comprised of 4 trials with a known target (Veliger positive sample from Canyon Lake with ~60 veligers/mL) and two known non-targets (samples from Calavaras Lake). Correct responses to the Canyon Lake sample in these trials were reinforced. The session

also included 2 trials known not to include a target (i.e., only samples from Calavaras Lake), in which a correct "all clear" response was rewarded. The remaining four trials were "unknown" in which one port presented the unknown lake to be screened with the remaining ports presenting known non-target lakes. These were "probe" trials and neither an alert nor an all clear were reinforced to avoid directly training a specific response.

The order of the lakes to be screened was randomized, but all canines screened the same lakes in the same order for logistical purposes of needing to change the samples in the olfactometer. One canine (Gilligan) did not participate after not showing sufficient detection of the "known positive" sample.

After canines completed the evaluation and data collection for Corpus Lake and Alan Henry Lakes, these two lakes were included as "known" negative lakes (based on historical data and absence of observed veligers microscopically). Canines received two sessions of training in which these two lake samples were considered "known negatives" and canines were reinforced for not alerting to them (one session following the assessment for Corpus and one session following the assessment for Alan Henry). After the training session, the lake was incorporated as a "known negative" for the remaining assessments. This additional training was done because canines previously had limited exposure to novel samples of negative lakes. This allowed canines to be provided with feedback on additional novel lakes, which is important to enhance generalization across novel samples (Lubow, 1974; Schrier & Brady, 1987).

eDNA Technology: Site Detection. The same method for vacuum filtration is used described in Objective 3. As described in Objective 2, an identical eDNA assay was used to screen all lakes with six replicates of all samples.

Results and Discussion

Microscopy. Using microscopy, 5 out of the 10 sites were determined to have *D. polymorpha* veligers present (Table 4). Using this traditional method proved difficult with the more turbid samples, which may have caused lakes where veligers were present to be incorrectly classified as *D. polymorpha* absent. This method was also time consuming and physically straining. Overall, this method took approximately four hours to completely survey the 10 sites under the microscope. It is possible that after a certain amount of time, the researcher identified macroinvertebrate larvae as veligers, resulting in a false positive. On the other hand, veligers could also have been identified as macroinvertebrate larvae, resulting in a false negative.

Canine Olfactory Detection. Figure 10 shows the probability of a canine responding to each of the tested lakes and the trained samples. The trained lakes all showed highly discriminative responding with the Canyon sample receiving a response on about 80% of trials in which it appeared and the trained negative lakes all receiving responses <25% of trials. This suggests canines can be readily trained to differentiate the lakes with and without veliger presence with reinforced training.

On the first evaluation of the untrained lakes in non-reinforced probe ("unknown") trials, canines' responses overall were generally high; however, a 50% probability of a response was used as a simple differentiator for canines responding to a lake as "positive" or "negative". Using this criterion, canines responded as "positive" to Ladybird, Walter E Long, Travis, Pflugerville, Georgetown, Austin, Choke Canyon, and Alan Henry. Canines evaluated Corpus and Granger as "negative".

Canines matched the eDNA results for 9 of the 10 lakes with the canines incorrectly identifying Choke Canyon as positive with neither microscopic nor eDNA evidence to suggest *D. polymorpha* presence (Table 4). Further, canines did categorize Lake Alan Henry as positive, however, based on sensitivity limits in Objective 2 and that microscopic evaluation did not reveal any veligers, this could potentially be considered an additional false positive.

eDNA Analysis. Extraction negative controls and qPCR NTCs tested negative for *D. polymorpha* eDNA. The standard curve y-intercept ranged from 23.4 to 23.9 cycles, slope ranged from –3.5 to –3.7, efficiency ranged from 85% to 91.5%, and R^2 ranged from .996 to 1. Across the 10 lakes sampled, *D. polymorpha* eDNA was detected at 7 of them with no detection at Granger, Corpus Christi Lake, and Choke Reservoir (Figure 11; Table 3). Rate of positive detection among water samples at each site ranged from 1 of 3 to 3 of 3, and within water samples, rate of detection among replicates ranged from 1 of 6 to 6 of 6.

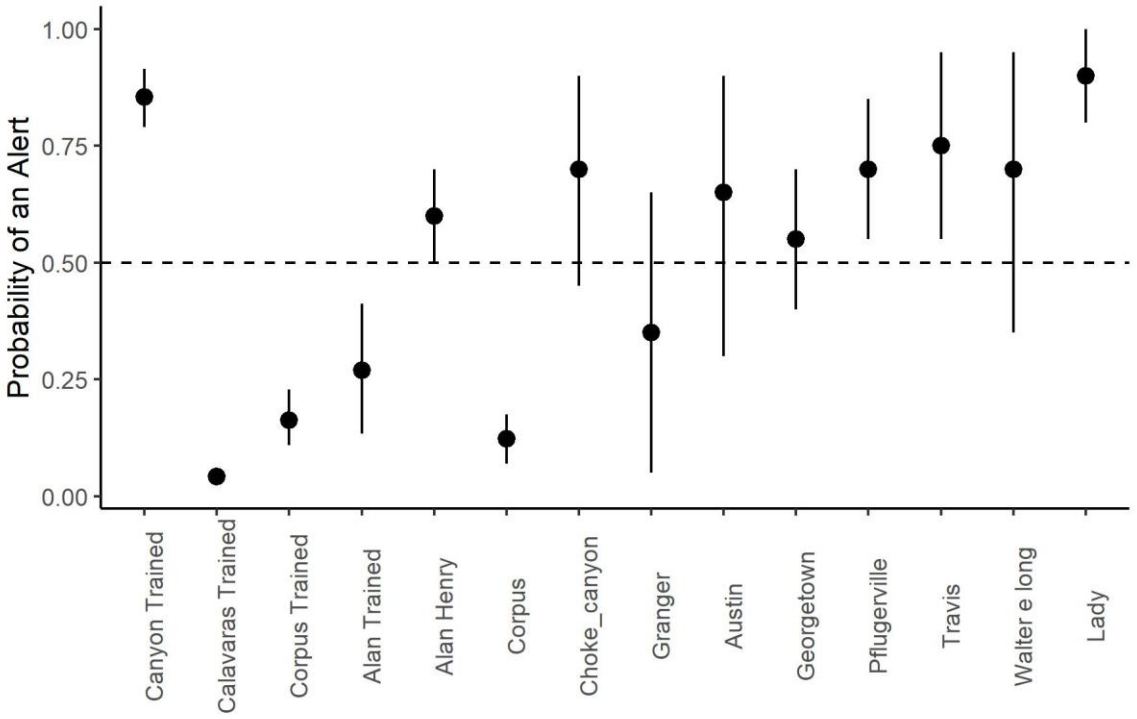

Figure 10. Canine Responding to each unknown lake and the explicitly trained samples. Points show the mean and error bars show the 95% confidence intervals.

All lakes screened by eDNA technology support TPWD's determined status of lakes apart from Lake Granger and Lake Alan Henry (Table 4). Lake Granger (labeled infested by TPWD) was determined to be negative with no detection from 3 of 3 samples and 6 of 6 replicates among all the samples. Lake Alan Henry (no detection by TPWD) was determined to be positive with detection in 1 of 3 samples and from 1 of 6 replicates among all the samples. This indicates a very low concentration of veligers or/and mussel eDNA. However, with no contamination in the non-template controls, it is unlikely this is a false positive.

Table 3. Summary of results from eDNA analysis on the ten different Texas lakes.

	Number of Sample Detections	Number of Well Detections	Average Ct	Average Standard Deviation
Lake Corpus Christi	0 of 3	0 of 18	0.00	0.00
Choke Canyon Reservoir	0 of 3	0 of 18	0.00	0.00
Lake Alan Henry	1 of 3	1 of 18	2.18	9.25
Lake Walter E. Long	3 of 3	18 of 18	35.02	0.46
Lake Lady Bird	3 of 3	11 of 18	23.53	19.33
Lake Austin	3 of 3	18 of 18	30.86	0.21
Lake Travis	3 of 3	18 of 18	30.23	0.26
Granger Lake	0 of 0	0 of 18	0.00	0.00
Lake Georgetown	3 of 3	18 of 18	31.48	0.73
Lake Pflugerville	2 of 3	12 of 18	23.82	17.44

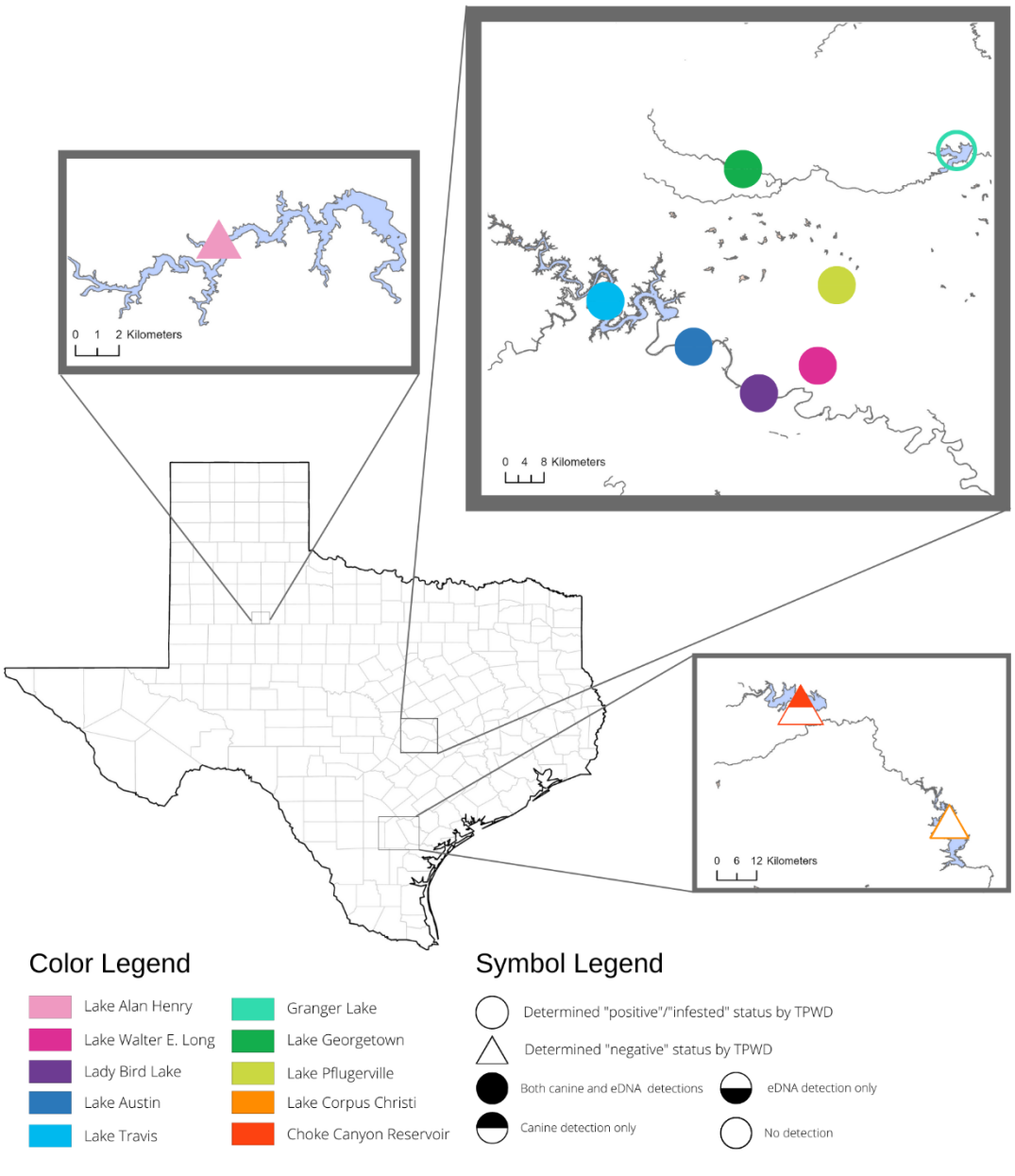

Figure 11. Comparison map showing published status by TPWD, results from eDNA technology, and results from canine detection. Canine detection results are based on ≥50% probability of detection.

Table 4. Summary of results from all detection methods utilized in Objective 4 and published status of lakes by TPWD. Canine detection results are based on ≥ 50% probability of detection.

	Texas Parks and Wildlife Division	Microscopy	Canine Olfactometry Detection	eDNA Technology
Lake Alan Henry	Negative	Not Detected	Detected	Detected
Lake Walter E. Long	Positive	Detected	Detected	Detected
Lady Bird Lake	Infested	Detected	Detected	Detected
Lake Austin	Infested	Detected	Detected	Detected
Lake Travis	Infested	Detected	Detected	Detected
Granger Lake	Infested	Not Detected	Not Detected	Not Detected
Lake Georgetown	Infested	Not Detected	Detected	Detected
Lake Pflugerville	Infested	Detected	Detected	Detected
Lake Corpus Christi	Negative	Not Detected	Not Detected	Not Detected
Choke Canyon Reservoir	Negative	Not Detected	Detected	Not Detected

Of the error canines made, canines were more likely to categorize a lake as positive than negative. This occurred for Choke Canyon Reservoir in which all other tests indicated a negative result. In addition, canines also categorized Lake Alan Henry as positive, although microscopically it was negative and only 1 of 18 eDNA replicates were positive. Although it can't be ruled out that D polymorpha volatiles were present, based on canine sensitivity limits in Objective 2, it seems more likely that this result would reflect a false positive result.

All technologies indicated Granger as negative despite the status of "infested". This could suggest that there has been a decline in *D. polymorpha* at this site, but the more likely lesson is that sampling location within a waterbody represents an important consideration for future research. All technologies were based on the same sample collection site. Thus, the negative result may reflect a negative result of the sampling site, rather than the lake. Future research would benefit from including multiple sampling sites of the same lake as an additional factor to compare technologies and identify optimal sampling parameters for the future.

Another consideration is that canines had a limited scope of training samples (lake sources) and time for training prior to assessment with several novel lakes. Due to necessary overlaps of canine availability and *D. polymorpha* veliger production, the time window for training was limited. Prior to assessment, canines had experience with veliger sources from two different positive lakes and two different negative lakes. When using canine detection across novel samples, it is ideal to have as many exemplars of positive and negative samples as possible (Bhatt et al., 1988; Essler et al., 2021; Hernstein et al., 1976; Schrier & Brady, 1987). Use of multiple exemplars of positive and negative samples is important to ensure canines are only identifying odor signatures associated with *D. polymorpha* veligers and nothing else. However, this is nontrivial due to physical limitations to access large reservoirs of similar size with differing *D. polymorpha* status within a radius. Future work may benefit from collection of samples across multiple states with collaborating partners to allow for greater access to more positive and negative samples to support canine training.

Notwithstanding, this assessment of canine performance is one of the more robust tests of canine performance as a detection technology to date. Frequently, canine performance is only assessed on samples used in training with known outcomes by the experimenter (Edwards et al., 2017) with infrequent test of canines' ability to generalize to novel untrained samples (Elliker et al., 2014). Additionally, frequently, detection assessments do not use operationally relevant frequency of targets and non-targets. In this case, samples were collected based on size and location within the testing region. Therefore, the lakes do represent an operationally relevant frequency of target and non-target samples. Additionally, the sample outcomes were unknown to the experimenters at the time of testing. Therefore, experimenters could not influence results one way or another nor provide canines with explicit feedback to train a response one way or another. Together, these procedures make the most robust assessment of the potential to use canines as a screening technology, with generally positive outcomes.

One important limitation to the canine results is that to provide canines with additional exemplars of negative lakes, canines were trained with a sample of Lake Alan Henry based on microscopy results obtained after the initial assessment. Microscopy results indicated the lake to be a negative sample, although eDNA did indicate a weak positive response (1 of 18 runs

returned positive). We view this limitation as minor given that Objective 2 demonstrated that canines sensitivity does require veliger presence, and it is unlikely with such low concentration of eDNA there may have been veliger associated volatiles present. Nonetheless, this limitation cannot be ruled out.

Objective 5: Conduct an economic analysis on eDNA and canine detection technologies based on measured accuracy, sensitivity, specificity, and cost per sample.

To assess the feasibility and potential usage of canines for the detection of Mussel veliger presence, and economic cost benefit analysis is important to assess. Objective 2 indicates that most canines need 10s to 100s of veligers/mL to accurately detect the target with greater than 75% accuracy. Objective 3 indicates that a single throw and pull of a plankton net is insufficient to provide the concentration necessary for adequate detection. Objective 4 indicates that if concentrated samples are taken from 10 "unknown" lakes, canines did overall correctly categorize a majority of the (9 out of 10 with respect to eDNA). Together, these results indicate that canines can be a potentially useful and efficient screening technology, although an odor concentration step is likely going to be necessary. Nonetheless, the concentration process used can be readily done in the field (no power requirements) and is quick. The longest part of the sampling process is the repeated use of the plankton net. However, it is important to note that we did not evaluate whether the plankton net itself is necessary.

Commercial canine screening/evaluation quotes for professional canine handlers were solicited from two organizations. $100/hr was provided as a standard hourly charge for canine services which covers all relevant canine care and maintenance. Analyzing the duration of a screening session, canines completed the 10-trial session providing 4 evaluations of an unknown lake in ~5 min. Adding 5 min of preparation times for the olfactometer setup to the canine screening time suggests an unknown sample can be processed in as little as 10 min for 1 canine. If a consensus of 2 canines is used, a sample can be processed and analyzed in 15 mins. This suggests that a 1-canine screening can be done economically for $17/sample or with a 2-canine consensus at $25/sample.

Prices of commercial eDNA detection services were also solicited from two environmental consulting companies. The first company offered tiered pricing depending on how many target species are screened per sample, with costs of $45/sample for single-species detection, $80/sample for detection of two species per sample, and $115/sample for three species detection per sample. Their fees also note that "discounts are available for large orders" and "same-sample replicates discounted $10." This pricing system demonstrates a key distinction between eDNA and canine surveillance; eDNA is multiplexable (i.e., multiple targets can be assayed at once) and scalable (i.e., the process becomes increasingly time- and cost-efficient as sample and target number increases). From our personal experience, the slight discount provided by the first company as the analysis transitions from single-species to multi-species

detection should be considerably larger, as much of the time and resource investment in the eDNA analysis process occurs in the DNA extraction process, and simply running additional PCR replicates for novel targets of the same DNA extraction should add mere dollars to the total cost (i.e. rather than nearly doubling the price). A second company didn't offer PCR-based single-species detection, but instead focused on next-generation sequencing, a process that simultaneously sequences all DNA in a sample and uses bioinformatic processes to sort and identify the provenance of each sequence recovered, typically from specific target groups such as microbes, fish, or vertebrates. Indeed, most commercial genetic monitoring companies have shifted their focus to offering these types of services since they are extremely scalable (i.e., 100 samples can be sequenced for same price as a single sample), and costs of such analyses continue to fall rapidly while accuracy and quantity of data produced increases. A second company charges $7200 for next-generation sequencing analysis of up to 48 environmental samples, which corresponds to $150 per sample for single-species detection, with the added data of all other DNA sequences cooccurring within the sample.

Conclusions

The establishment of non-native *D. polymorpha* in lakes throughout the United States has caused a significant ecological and economic impact. Detecting their presence in lakes and rivers before they establish a reproducing colony could help prevent further infestation in native lakes. This highlights the need for rapid and sensitive detection methods to facilitate mitigation actions. We compared canine detection and eDNA detection and evaluated their ability to detect *D. polymorpha* under a combination of laboratory and real-world conditions. Our results highlight the strengths and weaknesses of both detection technologies, allowing us to move one step closer to administrating an appropriate early detection protocol for *D. polymorpha*.

Objective 1: Training canines to *D. polymorpha* odor profile.

This study is the first to use canines to detect *D. polymorpha* veligers. Due to this, initial training and a high degree of success probability needed to be achieved before comparison of eDNA methods could begin. Canine training on *D. polymorpha* odor profile was successful in showing that canines can consistently detect veligers within a concentrated water sample. We found that canines were still able to detect samples where veligers were spiked into differing background water samples (i.e., distilled water, negative lake water with and without concentrated plankton). These trials indicate that canines can detect an odor signature associated with *D. polymorpha* in double blind controlled trials. With this initial success, we investigated canine limits of detection for *D. polymorpha* veliger presence and compared this to eDNA analysis.

Objective 2: Quantifying limits of detection of canine olfaction and eDNA analysis under simulated laboratory conditions.

Successful early detection methods should aim for high sensitivity to detect initial, low amount of *D. polymorpha* presence. Quantification limits determined that canine methods could detect veligers with a lower limit range of 0.825-313 veligers/mL, while eDNA has a detection limit of 0.026 veligers/mL. This suggests that eDNA has a greater sensitivity (30 to 12,000 fold greater from the most to least sensitive canine) and can determine the presence of *D. polymorpha* veligers and/or DNA in lower concentrations comparatively.

Canine training was done in a limited amount of time with a limited amount of lake samples. A longer canine training time with a greater variety of samples could greatly increase their sensitivity limits through perceptual learning mechanisms, but nonetheless, following the initial training, canines' detection limits were several folds lower than eDNA.

Objective 3: Determine eDNA downstream detection capability to *D. polymorpha* veligers of known mussel beds in rivers.

As suggested by Objective 2 and confirmed in continued assessments of canine detection, it was concluded that canines require a concentrated sample to detect the target odor. Canine methods for downstream detection were unavailable due to plankton nets not being suitable for river systems. However, sample collection optimization should be further researched for canine methods and thus determine canine's potential for downstream detection. Sample collection trials could be conducted using different sample collection tools such as traditional scoops with additional filtration recommended by Johnson et al 2019 or different styled nets that are more suited to shallow waters. Methods that prepare successful samples for canine detection should then be compared to traditional scoop methods for efficiency. In addition, canine detection accuracy from optimized collections should be compared with eDNA methods. If canine sample preparations become inefficient or complicated, this could limit the flexibility of canine detection to deep water bodies (where plankton nets can be used). In contrast, if a method proves viable for canine detection, this will strengthen the use of canine methods for rapid on-site detections.

Downstream detection using eDNA technology was capable of detecting *D. polymorpha* eDNA. *D. polymorpha e*DNA quantification illustrated an expected decreasing pattern of decreasing eDNA concentration as distance increased from known mussel beds upstream. eDNA was capable of detecting *D. polymorpha* eDNA at our furthest sample site with a distance of 14 river km from an infested lake. This indicates that eDNA methods are sensitive to low quantities of veligers and/or eDNA material. In terms of early detection, results at different sampling sites could suggest the source of *D. polymorpha* eDNA to be the transportation of veligers and/or DNA by river streams; established mussel beds throughout the river; a source of mussel introduction (i.e., recreational boats) into the river water body; or the downstream dispersal from an uninvaded lake. Further research needs to be conducted to interpret results of river detections and their implications for early detection methods.

Objective 4: Screen ten Texas lakes to compare microscopy, canine detection, and eDNA technologies.

Real-world samples were important to use to further compare canine and eDNA methods and to additionally give the opportunity to compare these methods with traditional and current methods (i.e., microscopy and TPWD determinations respectively). Using these methods to screen ten lakes, results suggested that microcopy was the least efficient and matched eDNA results for 8 out of ten lakes. The lower consistency of microscopy compared to other methods could be a result of several factors. First, the presence of lake material in the more turbid water samples obstructed the view of possible veligers. Additionally, microscopy has the possibility of misidentification that may contribute to false observations. In contrast, canines matched the results of eDNA for 9 of 10 lakes and matched microscopy for 7 of 10 lakes. Canines overall showed a higher probability to indicate microscopy and eDNA negative lakes rather than miss lakes. This propensity of false positives may be something that could be reduced or eliminated with more extensive training with examples of negative lakes that were not immediately available to the project team within the location limits. Nonetheless, future research could evaluate canine performance when assessed in areas in which lake status is more likely to be non-infested rather than invested (as was this case in the present study).

Together, both canine and eDNA detection results contradicted three of ten lakes statuses previously determined by TPWD. Statuses designated to lakes from TPWD are based on physical observation of adult mussels and veligers via visual confirmation by video imaging, scuba surveys, and microscopy.

Perhaps the most interesting result was the detection of *D. polymorpha* by both canine and eDNA methods in Lake Alan Henry. Lake Alan Henry is currently listed as 'Negative' by TPWD. Our eDNA results yielded a very low quantity of DNA within one of our three replicate samples. This suggests that Lake Alan Henry could be in the early, and most manageable, stage of infestation. The fact that both methods detected the lake as positive suggests that both methods could be comparable in early detection. However, further discussed in this objective, canine detection's determination is questionable due to the low concentration that was observed during microscopy and eDNA methods. This determination is further questioned by the higher sensitivity limits of canine detection determined in Objective 2 and canines' higher overall probability to respond to a novel lake as a positive rather than a negative. Thus, although the canine alert to Lake Alan Henry is intriguing and exciting for future research, the bulk of evidence collected within the project period suggests that this was more likely an incidental positive. This is further supported by the fact that when the Alan Henry sample was used as trained negative (based on microscopy results to provide canines more examples of negatives), canines did readily treat the sample as negative with minimal training. If a strong odor signature was available, we would suspect canines would require substantial training to

prevent additional alerts, which was not the case. Thus, although a majority of evidence suggests this was an incidental positive finding for the canines, it cannot be ruled out that in this circumstance there may have been a minor VOC profile indicative of possible veliger presence which caused the initial high probability of alert for the canine. Most importantly, the eDNA result, in combination with the canines' result, do suggest that Lake Alan Henry should be strongly considered as a target for additional testing and potential mitigation efforts. The positive signal obtained indicates that *D. polymorpha* presences are minimal and may be susceptible to mitigation.

Granger Lake's lack of detection (canine, microscopy and eDNA) from both methods also contradicted TPWD's listed status of 'Infested'. Both canine and eDNA methods could therefore prove useful for further monitoring of already infested lakes to provide an updated assessment. These assessments could be useful to management programs, for their resources can be more appropriately used for other lakes of concern. To support this suggestion greater sampling efforts of Granger Lake should be tested to determine the validity of method results. Sampling could include further concentration efforts or using multiple sample sites around the lake. Given that all three detection tools leveraged a sample from the same access point, it is possible the negative result was largely driven from obtaining a negative sample point. This suggests that additional research evaluating the number of sample points that may be necessary for evaluation to get robust detection would be a useful future direction. Downstream analysis in Objective 3, however noted eDNA detection in substantially distant downstream locations, which further makes the current lack of detection from eDNA interesting. Together these results suggest that exploring high density sampling at various access points could be a fruitful avenue to identify optimized sampling methods.

Choke Canyon Reservoir was the final lake that showed contradicting results. Along with TPWD status, eDNA methods and microscopy determined this lake as 'Negative' or 'Not Detected'. Canine methods in contrast determined the lake as 'Detected' with a high response value of about 80%. This was the only contradictory result made between canine and eDNA methods and reflects a canine error. This false positive may indicate that there was some overlap in the odor signature of Choke Canyon with that of the other samples that were veliger positive. Or, alternatively, the VOC profile of the Choke samples may simply have been more novel than other samples and canines responded to the novelty of the odor signature as a potential positive. These results nonetheless suggest that the canines required more exemplars of negative lakes as part of their training experience to refine their categorization of novel lakes. Future research could ensure using at least 10 positive and 10 negative lakes as part of the training phase to facilitate improved screening of novel unknown lakes. This was unfortunately not possible within the current scope given the few large lakes and reservoirs that were negative within travel distance of the study site.

For the present study, we used a simple cutoff of 50% alert rate to separate a "positive" from a "negative" response to a lake. Future work that may have access to a greater range of positive and negative lakes for training would likely produce greater separation or differentiation between positive and negative samples. For example, with the explicitly trained lakes, canines showed response rates >75% for positive samples and <25% for negative samples. If canines had more training samples, more of the unknown lakes may have fallen within these categorizations and would allow for more refined canine conclusions such as very likely positive, likely positive, likely negative or very likely negative. This refinement could be important to indicate which samples should be submitted for additional scrutiny and screening and for which samples may not be necessary.

Objective 5: Conduct an economic analysis on eDNA and canine detection technologies based on measured accuracy, sensitivity, specificity, and cost per sample.

From our economic analyses, we summarize that a cost of $25 per sample could support a screening protocol that would provide consensus of a team of two dogs. Per-sample costs of eDNA analysis based on commercial companies was higher, ranging from $45-150 per environmental sample, although from our personal experience, per-sample costs of eDNA analysis approach canine screening costs in an academic setting where profits are not included in the equation. Differences in sensitivity as noted in other sections of this report (i.e., particularly Objective 2) may make slightly higher per-sample cost of eDNA analysis more justifiable. However, the on-site capability of canine analysis exceeds that of eDNA analysis, for which on-site DNA extraction and PCR remain in their infancy (but see Doi et al. 2021) and typical analysis times measure several hours at a minimum, making canine detection an optimal choice where time is of the essence (i.e., screening vessels entering and leaving waterbodies).

Several distinctions between professional eDNA application and canine screening are notable, especially for a management agency or other group who may consider partially or completely processing their own samples. First, independent eDNA monitoring may be easier to support relative to canine detection because after the initial expense of purchasing infrastructure such as a PCR thermalcycler, upkeep is minimal and routine, especially compared to the daily housing, feeding, and other welfare activities (i.e., scheduled and illness-related veterinary visits) required for canine husbandry. The ability to analyze environmental samples for specific targets (i.e., *D. polymorpha*) depends on the existence of species-specific genetic assays in the peer-reviewed literature or other reports. In the case of the high-profile invasive species targeted in our study, many assays were available from which to choose (i.e., Peñarrubia et al. 2016, De Ventura et al. 2017, Gingera et al. 2017, Amberg et al. 2019, Blackbman et al. 2020), but other, less-studied species may require development and optimization of eDNA assays. This

process can require considerable investment of time and other resources in the collection of biological samples of target species, closely related species, and cooccurring organisms, as well as bioinformatic expertise and experience, and an iterative trial-and-error process of assessing species-specificity of candidate assays.

Overall, the economic comparison between eDNA and canine-based detection methods likely comes down to the purpose of sampling. For situations where results are needed rapidly, such as on-site monitoring of watercraft as they enter uninvaded lakes, canine detection represents the optimal method despite decreased sensitivity. Analysis of eDNA offers increased sensitivity (and additional data generation in the case of next-generation sequencing analysis) at the cost of processing time. The relative strengths of the two approaches may work well in tandem in a protocol beginning with bulk screening of multiple locations within a lake or multiple lakes to indicate which samples should be submitted for eDNA.

Overall Discussion

Canine detection of D polymorpha veliger presence in water samples was evaluated and confirmed. When screening a series of 10 novel "unknown" lakes, canine results matched eDNA on 9 of 10 and microscopy results on 7 of 10 (microscopy and eDNA matched 7 of 10). Detection canines, however, did show substantially poorer detection limits compared to eDNA (30-12,000-fold poorer for most and least sensitive canines) and canines did require additional water filtration and veliger concentration steps for adequate detection levels.

Nonetheless, canine evaluation was rapid (~5 min per lake of canine time) and cheap (~$25 to get a consensus result of 2 canines). This reflects at least $20 savings over eDNA. Given, however, that poorer resolution of higher probability for canines to make a false positive, these results suggest that both tools could be used in conjunction to optimize costs and testing information. For example, management organizations could use the cheaper and rapid cost of a canine evaluation to sample many points within a lake or across many lake bodies to identify which samples have the highest probability of detection. After a rapid and cheap canine screening, samples with ambiguous status or samples with the highest probability of being positive can be submitted for eDNA confirmatory testing. By combining tools, canines can provide a cheaper lower quality screening with higher throughput that identifies the best candidate samples for confirmatory and quantification via PCR. Future research projects could leverage these two tools in an experiment to mostly cost effectively map highest concentrations of veliger populations within a lake by allowing canine screening to efficiently screen many samples within the lake and eDNA providing quantification of the most strongly detected samples.

Due to the nature of novel canine detection in aquatic targets, further adjustment of protocols needs to be explored to determine optimized sampling and optimized training techniques. This includes a longer training period to determine if odor sensitivity and detection probabilities can be increased. In addition, sample preparation optimization should be designed specifically for canine detection in areas where plankton nets cannot be used. Additionally, leveraging broader communities and locations to provide more non-infested samples would be an important next step to refine canine performance by providing more examples of negative lakes and would most likely improve canine categorization (Astley & Wasserman, 1992; Schrier & Brady, 1987).

The present effort has been to date the most thorough evaluation of canine detection for invasive mussel veligers by (1) robustly assessing detection performance in double blind computer controlled conditions for detection of veligers in various water backgrounds, (2) assessed canine limits of detection with comparison and verification of quantification via eDNA, (3) established lack of detection using non-concentrated samples, and (4) demonstrated canine performance in concordance with eDNA and microscopy when screening novel unknown lakes for which the canines were not previously trained. Overall, canine results are positive although demonstrating poorer sensitive than eDNA. These results suggest that with further study, optimized screening protocols for mussel intrusion can be developed that maximize data collection while minimizing cost leveraging canines as a bulk screening tool where eDNA provides final confirmatory results of a smaller subset of samples indicated by canines.

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