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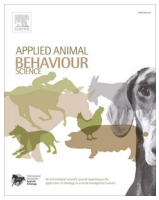

How low can they go: A comparison between dog (*Canis familiaris*) and environmental DNA detection of invasive koi carp (*Cyprinus rubrofuscus*)

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ABSTRACT

Carp (*Cyprinus* sp.) are a highly invasive fish that pose a significant threat to freshwater ecosystems worldwide. At high biomasses (i.e., ≥100 kg/ha), the benthic feeding behaviour of carp can have deleterious ecosystem effects, often changing clear, macrophyte dominant waterbodies to turbid-phytoplankton dominant ecosystems. To prevent carp from reaching ecologically harmful biomasses, early detection (i.e., before population establishment) and rapid control actions are vital. Boat electrofishing, netting, and environmental DNA (eDNA) are commonly used to survey for carp, but these methods can be expensive or time inefficient when fish densities are low. Scent-detection dogs have proven efficacious at detecting terrestrial and aquatic species and could represent an efficient method for early detection of carp invasions. Here, we investigated a dog's sensitivity and specificity to carp scent using a multiple probe design experiment. The dog was trained to use an automated carousel independently of its handler and assessed water samples from aquaria containing either no fish scent ($n = 3$, non-target), goldfish (*Carassius auratus*) scent ($n = 5$, non-target) or carp scent ($n = 9$, target). The goldfish samples and six of the target samples were presented to the dog at a standard fish biomass concentration of 15.5 mg/L. The remaining three target samples (probes) were systematically diluted to determine the dog's detection threshold. Results showed that the dog could detect carp housed under laboratory conditions down to a dilution equivalent biomass of 9.3 kg/ha (i.e., 0.5 mg carp/L), which is well below the likely biomass threshold of carp known to cause significant ecological impacts. The dog's scent-detection performance was then compared with eDNA, an existing survey method. Quantitative PCR conducted on DNA extracted from laboratory aquaria filtrate revealed that the species-specific primer could detect carp at 9.3 kg/ha, but amplification rates at this dilution were low, as were all dilutions below the limit of quantification (≈160 kg/ha). These findings suggest that dogs could provide an accurate and highly sensitive method invasive fish detection. However, before deployment as a carp surveillance method, dogs' performance on water samples collected from natural aquatic systems (i.e., lakes, ponds, rivers) needs to be evaluated. To our knowledge this is the first published study of its kind comparing a dog's sensitivity to eDNA, and investigating the potential utility of dogs as an invasive fish detection method.

1. Introduction

Common carp (*Cyprinus carpio*) and the closely related koi carp (*Cyprinus rubrofuscus*) are highly invasive fish species that have been introduced to freshwater ecosystems worldwide (McDowall, 1996; Hicks and Ling, 2015). Often referred to as "ecosystem engineers," carp are known to modify aquatic systems through their foraging behaviour (Matsuzaki et al., 2009; Weber and Brown, 2009; Gozlan et al., 2010). Carp forage in bottom sediments uprooting aquatic vegetation and re-suspending nutrient laden sediment into the water column (Weber and Brown, 2009; Bajer and Sorensen, 2015; Qiu et al., 2019). At high densities (≥100 kg/ha), this feeding activity can cause major reductions in water quality, decreasing food and habitat available for native species (King et al., 1997; Chumchal et al., 2005; Driver, 2005; Bajer et al.,

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2009). Once carp populations have established, they can be difficult and costly to eradicate (Lougheed et al., 2004; Weber and Brown, 2009; Hicks et al., 2015). Therefore, it is vital that new incursions of carp are detected early, when population densities are low (i.e., <10 kg/ha), and management actions can be undertaken towards control or eradication.

Environmental DNA (eDNA) is a non-invasive survey method increasingly used to detect and monitor species (Rees et al., 2014). Species can be identified from DNA shed into the environment via processes such as reproduction (e.g., gametes, larvae), metabolism (e.g., faeces, urine), growth (e.g., scales, skin etc.), and decomposition (David et al., 2021). Compared to traditional fish detection techniques such as electrofishing and netting, eDNA has demonstrated increased sensitivity, and is often more time- and cost-efficient when fish densities are low (Jerde et al., 2011; Rees et al., 2014; Wilcox et al., 2016; Evans et al., 2017; Hinlo et al., 2017; Piggott et al., 2021). Despite these advantages, abiotic (e.g., flow rate, UV radiation, salinity, temperature, pH) and biotic factors (e.g., extracellular enzymes and microorganisms) can influence the amount of detectable DNA in a water body. Both water quality and the volume of water filtered can influence the quantity of DNA in the sample. In addition, primer sensitivity and specificity, DNA mutations and PCR inhibitors (e.g., humic acids) can prevent successful polymerase chain reactions (Ficetola et al., 2008; Herder et al., 2014; Strickler et al., 2015; Thomsen and Willerslev, 2015; Caza-Allard et al., 2022). Given these limitations, novel methods of invasive species detection should be investigated.

Dogs have an advanced olfactory system that is between 10,000–100,000 times more sensitive than humans (Walker et al., 2003; Walker et al., 2006). As such, they have been used for a wide range of tasks including the detection of plant and animal species (Browne et al., 2006; Beebe et al., 2016). Dogs detect organisms from the volatile organic compounds (VOCs) released into the environment. Given that dogs can detect low concentrations of VOCs, are highly trainable, and can cover large areas, they often outperform other detection methods (Concha et al., 2019; Grimm-Seyfarth et al., 2021). For example, Thompson et al. (2020) planted bilby (*Macrotis lagotis*) scats in four locations and found that dogs detected 98.9% (89/90) while human searchers only found 6.7% (6/90). The researchers also found that dogs were more efficient, finding the first scat in significantly less time than humans (dogs: 72.8 s; humans: 361.2 s). Although most scent detection research has focussed on dogs' detection of terrestrial species, they also appear to be effective for detecting aquatic organisms.

Rolland et al. (2007) trained dogs to detect North Atlantic right whale (*Eubalaena glacialis*) scat from a boat and compared their relative sampling efficiency to opportunistic scat collections by human searchers. Faecal sample collection rates were four times higher using dogs than opportunistic methods. DeShon et al. (2016) demonstrated that under controlled laboratory conditions, dogs could detect larvae of the invasive quagga mussel (*Dreissena rostriformis bugensis*) at concentrations similar to those found in natural aquatic systems. Given dogs' ability to detect a wide variety of target scents, including highly cryptic species, they could also be used to detect incursions of invasive fish in water samples retrieved from the field.

To be an effective early detection tool, dogs need to be able to detect carp before the population becomes too large to effectively manage. In this experiment, sensitivity and specificity to koi carp scent housed in laboratory aquaria were compared to these same measures obtained with eDNA. It was hypothesised that the detection dog and eDNA would be able to discriminate koi carp from non-target samples at low biomass levels. If successful, dogs could represent an effective early detection method for invasive fish.

2. Methods

2.1. Subject

One dog, Ruby (female Labrador retriever/border collie cross, 8 years old), was selected from 13 initial candidates to participate in a multiple-probe design experiment. Approval for this experiment was obtained from the University of Waikato Animal Ethics Committee (protocol #1013).

2.2. Fish housing and sample collection

Three identical 195-L high density polyethylene tanks with fitted lids to prevent cross-contamination were used for fish housing at the University of Waikato, New Zealand. Tanks contained either no fish (control tank, non-target scent), goldfish (non-target scent), or carp (target scent). Under standard conditions all tanks received a continuous supply of dechlorinated tap water (0.5 L/minute), and oxygen levels were maintained using aerators. Twenty-four hours prior to water sample collection, the water supply to all tanks was halted, and each tank was drained and cleaned with a designated cleaning pad. All tanks were then flushed and refilled with water. The tanks were filled to a level where fish biomass equated to 15.5 g/L to standardise sample potency, fish were then held without flow-through, while the control tank remained on flow-through. After 24 h, water samples were collected from each tank using tank-specific glass beakers and bottles to avoid cross-contamination. Non-target samples were collected first (control, then goldfish), followed by the target samples (carp). To minimise residual odours and cross contamination, all glassware was washed in either sample-specific 10% v/v hydrochloric acid (HCl) baths (i.e., collection bottles) or concentrated nitric acid (HNO_3; i.e., beakers), followed by triple rinsing with reverse osmosis water and oven drying at 40 °C. Acid baths were replaced twice during this experiment.

During the probe experiment, a deep clean of all the experimental tanks was performed at the start of each month. Fish were removed from their tanks and placed in holding tanks. The experimental tanks were then drained, scrubbed with a disposable cloth, and sprayed with 10% v/v hydrogen peroxide to degrade residual organic matter. After 24-hours of flow-through water, new groups of goldfish and carp were transferred to the experimental tanks. New carp and goldfish were introduced every month over the duration of the probe experiment (15 weeks; 4 fish changes) to ensure the dog was generalising carp/goldfish scent and not responding to scent from individual specimens. All equipment used for fish capture (nets, buckets, etc.) was sprayed with 10% v/v hydrogen peroxide and allowed to air dry following each tank cleaning to mitigate cross-contamination.

2.3. Scent-detection apparatus

Scent-detection testing was conducted in an experimental room (dimensions: 3.2 m x 4.3 m) containing a fully automated scent-detection apparatus (SDA) at the Scent Detection Research Group facility at the University of Waikato, New Zealand. The SDA allowed the dog to perform scent-detection trials without the researcher being present in the room, thereby minimising the influence of human subjectivity or cueing. Briefly, individual water samples, either target (carp) or non-target (no fish or goldfish), were placed into one of 17 removable aluminium segments on a rotating carousel. A stainless-steel lid was placed on top of the segments to encase VOCs released from the water samples. A flap at the front of each segment allowed the dog to insert their nose and sniff the sample through a 10 cm port at the front of the apparatus. When assessing samples, infrared beams behind the port were broken initiating an audible "beep", and the dog's sniffing response was recorded by custom software. On non-target samples, the dog was required to assess the sample (i.e., hold their nose in the port/segment) for a minimum response duration of 501 ms and then press a switch positioned on the right-hand side of the apparatus, which rotated the carousel, presenting the dog with the next sample. On target samples, a pre-specified response duration (e.g., 4 s, but lower values were used during training) had to be met before the behaviour was reinforced with kibble via an automatic feeder positioned 2 m from the SDA. Both the

apparatus and the feeder were controlled by custom software outside the experimental room. A detailed description of the design and operation of the SDA is provided in Edwards (2019), and additional information about the performance of dogs using the apparatus with standard chemical samples is provided in Edwards et al. (2022).

2.4. Dog training

The dog was trained to approach and consume kibble from the automated feeder, initially operated by the researcher using a remote control. Once the dog began to reliably approach the feeder within 3 s of food delivery, it was trained to approach the SDA and place its nose in the sample port using the method of shaping (Skinner, 1975). During this phase, all SDA segments contained glass jars with 100 mL tank water with a carp biomass of 15.5 g/L, and reinforcement was delivered by the experimenter using the remote feeder each time the infrared beam was broken. Once the dog was reliably placing their nose in the sample port, the procedure was adjusted by programming the apparatus to only allow the feeder to release kibble after a response duration of 500 ms. Each time the dog assessed all 17 samples (hereafter, session) without prompts the response duration requirement was gradually increased until it reached 1.5 s. When the dog was consistently responding for 1.5 s, it was trained to press the switch on the side of the SDA to advance the carousel to the next sample. Once it successfully pressed the switch 10 times unprompted, discrimination training commenced and non-target water samples containing no fish scent were added to every second segment. During this stage of training, the experimenter stood to the right of the apparatus and, if necessary, prompted the dog to press the switch after assessment of a non-target sample. Prompts were progressively phased out until the dog was operating the apparatus independently. Once autonomous operation of the apparatus was achieved, the researcher gradually removed themselves from the room progressively increasing the dog's required response duration from 1.5 s to 4 s, followed by increasing a session to two full clockwise rotations of the sample array (i.e., all 17 samples were assessed twice). Goldfish water samples were introduced as another non-target scent and the sample order randomised. This resulted in a scent arrangement of seven carp samples, five control water samples, and five goldfish samples. Goldfish were chosen as an additional distractor scent for two reasons: (1) it is a close relative of carp so was thought to be a greater test of the dog's discriminative abilities, and (2) carp and goldfish commonly co-exist in freshwater ecosystems and thus discrimination is vital.

When the dog assessed a target sample, either a hit or a miss was recorded. Hits occurred when the dog held its nose in the port for the required 4 s response duration, this action was then reinforced. Misses were recorded when the dog broke the infrared beam but did not hold its nose in the port for longer than 4 s, for which there was no reinforcement. When assessing non-target samples, correct rejections were recorded when the dog broke the infrared beam for $>$ 500 ms but no longer than 4 s, followed by pressing of the switch to advance the carousel. A false alarm was recorded when the dog broke the infrared beam for $>$ 4 s, falsely indicating that the sample was positive. The dog was also required to press the switch after a false alarm.

The final step of training involved diluting fish samples in a stepwise manner. Each time the dog met accuracy criteria of $\geq$ 80% hit rate for target samples and $\geq$ 80% correct rejection rate on non-target samples (no fish scent and goldfish scent, individually) for four consecutive sessions, the fish samples were diluted by 50% in the following session. Once the dog had met criterion at a dilution equivalent to a biomass of 15.5 mg/L (hereafter, referred to as baseline concentration), it was considered fully trained. This concentration was chosen as the training endpoint as it is analogous to an areal biomass of 310 kg carp/ha, (based on a 2 m deep water body) which represents a biomass of carp commonly found in lakes within the Waikato Region of New Zealand (Hicks et al., 2015; Tempero et al., 2019).

2.5. Experimental design and procedure

Once trained, the dog participated in a multiple probe design experiment to evaluate its sensitivity to carp scent. Nitric acid (67% HNO_3) washed glass jars filled with aquaria water containing either no fish scent (n = 3; non-target), goldfish scent (concentration: 15.5 mg/L; n = 5; non-target), and carp scent (n = 9; target) were placed inside segments on the SDA. Six of the carp aquaria water samples, contained the baseline concentration of 15.5 mg/L; while the remaining three samples contained diluted concentrations of carp aquaria water (hereafter, "probes;" Table 1). Once in place, a stainless-steel lid was placed on the segments, and the samples were allowed to stand for 20 min to allow for release of VOCs before the session began. Each time the dog met an accuracy criterion of $\geq$ 80% hit rate on baseline and probe samples and a combined correct rejection rate (no fish + goldfish) of $\geq$ 80% on non-target samples for 2/3 sessions (one session = two full rotations of 17 samples), the probe samples were diluted by 50%. Hits, misses, correct rejections, and false alarms were recorded, as outlined in Section 2.4 (Dog training). All hits on target samples (probe or baseline) were reinforced. Four sessions were conducted per day, with a new set of randomised samples introduced after the first two sessions. The experiment was terminated after 45 sessions without meeting accuracy criterion with a given dilution.

2.6. Environmental DNA

Comparative, laboratory-based environmental DNA detection of koi carp was undertaken by performing serial dilutions on the same sample water assessed by the dog, ranging in biomass concentration from 15.5 g/L to 0.5 mg/L. Following serial dilution with ultrapure water, samples were filtered through self-preserving eDNA filters (5 μm size cut-off; Smith Root Inc., Vancouver, USA). Relative fish biomass in each sample was calculated based on initial biomass concentration and volume of water filtered. The control samples of dechlorinated tap water were also tested at various filtered volumes up to 10 L.

Filters were stored at room temperature prior to transportation to the Trace DNA Laboratory, University of Canberra, Australia where eDNA extractions took place within a designated low-copy DNA extraction room. Samples were extracted using a modified Qiagen DNeasy© Blood and Tissue Kit extraction protocol. The filters were submerged in a lysis solution consisting of 360 μL of ATL buffer and 40 μL of Proteinase K, followed by incubation for 1 h at 56 °C. After a brief vortex, 400 μL of 100% ethanol and 400 μL of AL buffer were added to each submerged sample. Two centrifugation repetitions of 8000 rpm for 1 min were completed to load a total of 1200 μL of sample supernatant into the mini-spin columns. The samples were then washed by loading 500 μL of AW1 buffer, centrifuging at 8000 rpm for 1 min, then loading 500 μL of AW2 buffer, and centrifuging at 14,000 rpm for 3 min. The extracted DNA was then eluted in 200 μL of ultra-purified deionised water. Two negative

Table 1
Fish biomass concentrations and equivalent areal biomass used to determine dog sensitivity to koi carp scent. Equivalent areal biomass assumes a 2 m deep waterbody.

Sample type	Fish biomass concentration (mg/L)	Equivalent areal biomass (kg/ha)
No-fish	–	–
Goldfish	15.5	310.6
Carp (baseline conc.)	15.5	310.6
Carp (probes)		
Dilution 1	7.7	155.4
Dilution 2	3.9	77.7
Dilution 3	1.9	38.9
Dilution 4	1.0	19.4
Dilution 5	0.5	9.3
Dilution 6	0.2	4.7

extraction control samples were also included to monitor for potential contamination during the eDNA extraction process.

2.7. Assay optimization

A species-specific TaqMan assay designed and validated by Furlan and Gleeson (2016) to detect common carp (*C. carpio*12 s) was used in this study. The assay targeted and amplified a 73 bp length fragment of mitochondrial DNA in the 12 s gene region of the common carp mitochondrial genome (Table 2). The assay was optimised at the University of Canberra to determine suitable reagent volumes and cycling conditions. Only sample optimisation was required for this assay as, in addition to amplifying common carp DNA, which it was designed for, it had also been proven to amplify koi carp DNA. Optimization of the sample involved running a single replicate of neat samples and a 1:10 diluted version of that sample against each other in a qPCR run to determine which version ran more effectively and gave the best results. An eDNA sample collected from a previous project, known to contain common carp DNA, was also used as a positive control for the assays, along with a genomic DNA positive control at a 1:100 dilution.

2.8. Quantification

Synthetic oligonucleotide standards were used during the initial stages of the project to check the validity of the assay and to ensure amplification of DNA. Following this, they were used as a standard in a dilution series of 1,000,000 copies/μL to 10 copies/μL to determine the threshold and limits of the assay. The limit of quantification (LOQ) can be defined as the lowest quantity of eDNA in a sample that can be confidently quantitatively determined. The limit of detection is the lowest concentration of DNA that can be confidently detected and can be used to show the tested assay's sensitivity in regard to detecting the target species' DNA (Klymus et al., 2020).

2.9. Real time quantitative polymerase chain reaction (qPCR)

The qPCR reaction mix consisted of 10 μL of Environmental Master Mix 2.0 (Life Technologies), 1 μL of 10 μM of *C. carpio*12 s-F, 1 μL of 10 μM of *C. carpio*12 s-R, 1 μL of 10 μM of *C. carpio*12 s-P, 2 μL of ultra-purified deionised water (Invitrogen), and 4 μL of template DNA to make a total volume of 20 μL. The PCR master mix and DNA were dispensed into 0.1 μL strip tubes and samples were run once in replicates of six. The samples were run with positive controls consisting of three replicates of each oligonucleotide concentration, non-template control (NTC), and the two negative extraction controls. The samples were run using the ViiA™ 7 Real-Time PCR system (Applied Biosystems) at cycling conditions of 50°C for 2 min, 95°C for 10 min, followed by 50 cycles of 95°C for 10 s, and 60°C for 20 s. A detection was considered positive if there was an exponential phase at any point during the 50 reaction cycles and amplified above the amplification threshold. All samples identified as positive amplifications were sent to The John Curtin School of Medical Research at the Australian National University for Sanger Sequencing.

2.10. Data analysis

Scent-detection sensitivity values were calculated for each target sample (baseline and probe) and specificity values were calculated for the two non-target samples, both individually and collectively (i.e., correct rejection per non-target type and combined correct rejection = goldfish + no fish). Sensitivity was calculated as the proportion of correctly indicated target samples from the total number of target samples, and specificity as the proportion of correctly rejected non-target samples from the total number of non-target samples. In addition to evaluating the dog's overall performance (i.e., sensitivity and specificity during all sessions completed by the dog), we also assessed the dog's performance in the first and second session to determine if repeated exposure increased the dog's detection performance. Finally, to determine if the dog's performance on probes varied as a function of accuracy, sensitivity and specificity, values were also calculated from sessions where a criterion of a hit rate on baseline samples of $\geq$ 80% and a combined correct rejection rate of $\geq$ 80% was achieved. All graphical and statistical analyses were performed using GraphPad Prism (Version 9).

3. Results

3.1. Scent detection dog performance

In total, 106 sessions over 29 testing days (15 weeks) were conducted, taking an average of 7 min to complete a session. The experiment was stopped at the 0.2 mg carp/L probe dilution without the dog meeting accuracy criterion after 46 sessions. Comparisons between the first and second session (Figs. 1A and 1B, respectively) revealed that the dog's hit rate on target samples, and correct rejection rates on non-target samples, generally increased in the second session. The most marked increases in detection performance were seen in the dog's correct rejection rates.

Evaluation of the dog's overall performance revealed that the hit rate on baseline carp samples remained high and relatively stable across all dilutions (range = 87.7–95.1%). The hit rate on probes was more variable, ranging between 68.1% (1.9 mg carp/L) and 92.8% (7.7 mg carp/L). No obvious trend between the dog's detection performance and probe dilution was observed (Fig. 1C). In general, the dog's correct rejection rate on no-fish samples was higher than the correct rejection rates on goldfish samples. The lowest combined correct rejection rate (62.2%) was recorded on the final probe dilution (0.2 mg carp/L) (Fig. 1C).

Of the 106 sessions, only 31 (29%) were completed with a hit rate on baseline samples and combined correct rejection rate of $\geq$ 80% (hereafter, high accuracy sessions) (Fig. 1D). When data from these high accuracy sessions were evaluated (Fig. 1C) it was evident that the hit rate on probes, regardless of the dilution, did not increase or only marginally increased relative to the hit rate in all sessions.

3.2. Environmental DNA

Calibration of the assay using synthetic oligonucleotides gave good linearity over the range of 10^6 to 100 DNA copies per μL of sample,

Table 2
Details of the assay used in the qPCR master mix for the testing of carp eDNA samples, targeting a 73 bp fragment of mitochondrial DNA in the 12 S gene region. From: Furlan and Gleeson (2016).

Assay	Label	Sequence (5′–3′)	Fragment length (bp)
Species–Specific Assay: Common carp (*Cyprinus carpio*)	*C.carpio* 12 s-F	CAAACTGGGATTAGATACCCCACTAT	73
	C.carpio 12 s-R	CTGGCGGACATCTAATTGTAGC	
	C.carpio 12 s-Probe	(FAM)-CAGCCGTAAACTC-(MGB)	
	C.carpio 12 s-Synthetic	CAAACTGGGATTAGATACCCCACTATGCTCAGC CGTAAACTCAGACATCCAGCTACAATTAGATGT CCGCCAG	

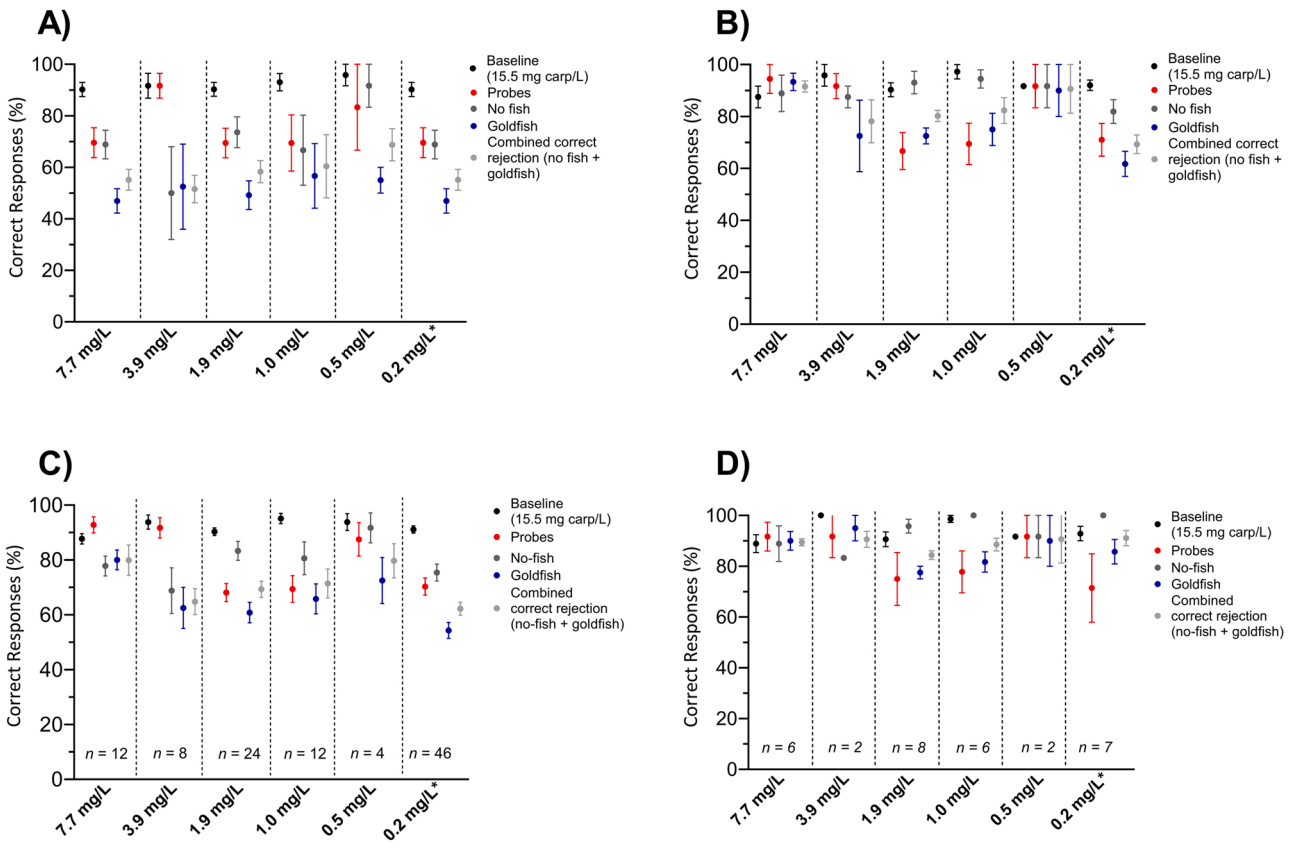

Fig. 1. The detection dog's (A) hit rate on baseline and probe samples, correct rejection rate on goldfish and no fish samples, and combined correct rejection rate on both non-target samples (± standard error) in the first session performed by the dog on each sample array, (B) second session, (C) across all sessions, (D) sessions where hit rate on baseline samples and a combined correct rejection rate of ≥ 80% was achieved (high accuracy session). The relative biomass of carp per probe dilution is displayed on the x-axis. The number of sessions (*n*) the dog took to meet the accuracy criterion and where the dog was performing accurately (i.e., hit rate on baseline samples and combined correct rejection rate of ≥80%) are also provided on graph C and D, respectively. * After 46 sessions the dog had not met criterion at this dilution.

although PCR success rate significantly declined below 1000 copies/µL (Fig. 2). Limit of quantification, based on a 90% likelihood of a positive PCR reaction, was determined to be 1300 copies/µL equating to a DNA concentration in water of 260,000 copies/L, assuming an extraction volume of 200 µL from the filter.

Comparison of normalized fish tissue biomass per litre with the qPCR CT values determined by the *C. carpio*12 s assay showed a reasonably linear relationship for biomass values above 10 mg/L, with the average CT value reaching 40 cycles at approximately 15 mg/L (Fig. 3) for 1 L of filtered water. Although it was possible to detect carp DNA in samples at much lower relative biomasses (as low as 0.46 mg/L), the PCR success rate was lower and positive samples were generally derived from larger volumes of filtered water (either 2 or 4 L). The *C. carpio*12 s assay also amplified samples containing goldfish DNA but with far lower efficiency (Fig. 3). No carp DNA was detected in any samples of control dechlorinated tap water up to filtered volumes of 10 L.

Comparison of calculated DNA copies per litre of filtered water with

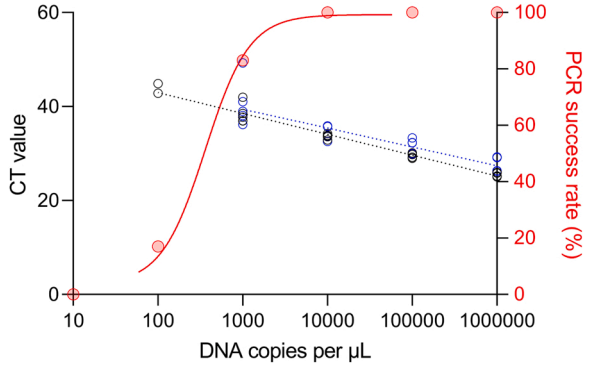

Fig. 2. Calibration of qPCR against synthetic oligonucleotide concentration. Two qPCR calibrations were undertaken: blue and black open circles respectively. Number of DNA copies is the copy number per uL in sample added to the PCR: a total of 4 µL was included in the pCR reaction. PCR success rate is the cummulative success rate from both runs (red dots and line).

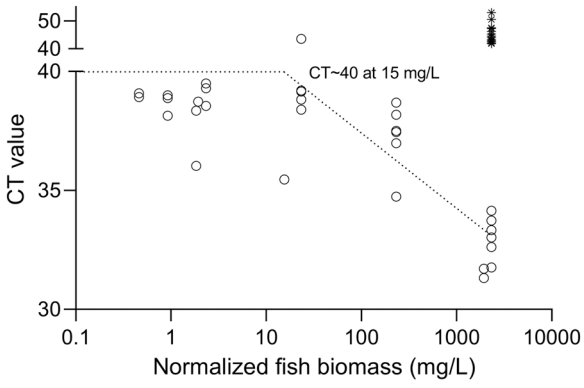

Fig. 3. qPCR CT values compared with relative fish biomass using the *C. carpio*12 s assay for carp (open circles) or goldfish (asterisks). CT values for samples below 10 mg/L are generally for samples with filtered water volumes greater than 1 L.

normalized fish biomass per litre revealed a limit of quantification for carp biomass of approximately 8 mg/L, based on a limit of quantification of 260,000 copies/L (see above; Fig. 3). A relative biomass of 8 mg/L corresponds to an environmental biomass of 160 kg/ha in a shallow lake with an average depth of 2 m. (Fig. 4).

4. Discussion

This is the first published study to investigate the potential utility of dogs as a detection tool for invasive fish, and to compare the sensitivity of dogs to eDNA. The results suggest that dogs are capable of reliably detecting carp odour at a dilution equivalent to 9.3 kg carp/ha (i.e., 0.5 mg carp/L) when presented with water samples from laboratory aquaria, which is similar to the sensitivity levels achieved by eDNA in this study. This level of biomass is approximately equivalent to 2–4 adult carp/ha (based on a 2 m deep waterbody), a population density feasibly managed before significant environmental effects. This indicates that, with further research, dogs have the potential to be used as an early detection method for invasive freshwater fish species.

While the detection dog could accurately detect carp at low biomass concentrations it was still susceptible to false positive (i.e., carp odour absent but incorrectly identified as present) and false negative (i.e., carp odour present but not detected) errors. False positive errors (false alarms) occurred on both non-target sample types but were more common with goldfish samples. This indication bias is not surprising given that carp and goldfish are sufficiently related to be able to produce hybrid offspring and thus likely have similar scent profiles (Chistiakov and Voronova, 2009; Banks et al., 2010; Hernández-Roldán et al., 2014; Warner et al., 2018). False positive errors could have also been due to cross-contamination, or the high ratio of target and non-target samples (9 targets to 8 non-targets; 53% chance of a target sample) (Lazarowski et al., 2020; Edwards et al., 2022). For example, Edwards et al. (2022) demonstrated that dogs' false alarm rates tend to increase with target sample prevalence, suggesting that if a lower proportion of target samples was used in this study (i.e., ≤6 targets), the number of incorrect indications on non-target samples could have been reduced.

False negative errors (misses) were expected to rise as sample potency decreased, but this trend was not evident in the dog's overall or "high accuracy" results. Instead, there was an increase in misses at intermediate probe dilutions (i.e., 1.9 mg carp/L and 1.0 mg carp/L). This change in detection performance was likely due to the dog experiencing minor health issues and thus intermittently attending sessions whilst these probe dilutions were being tested.

Capture-based methods such as netting or electrofishing can take days or even weeks to gain a positive detection when fish densities are low (e.g., Jerde et al., 2011). Environmental DNA is often much more efficient; although it may take several hours to process a single set of samples, and the processing costs per sample can be relatively expensive (Jerde et al., 2011; Evans et al., 2017). Using the methodology outlined in this study, we demonstrated that a dog could accurately assess 17 water samples, twice, within minutes (average session time = 7 min). Such efficiency suggests that if water samples were sent into the laboratory for evaluation by dogs, dogs could be more time and thus cost efficient than current detection methods.

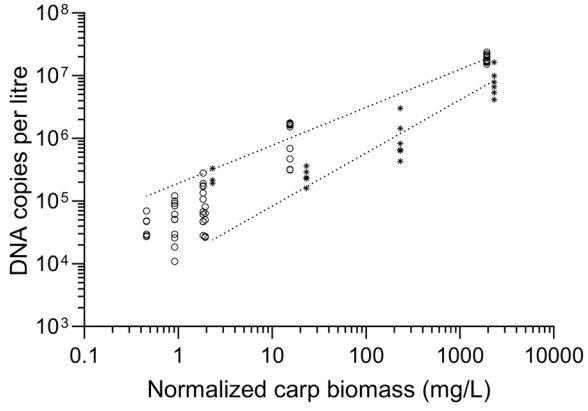

Fig. 4. Comparison of normalized fish biomass and DNA copy number calculated from qPCR. Results include two separate fish biomass dilution series (open circles and asterisks, respectively).

Environmental DNA was found to be capable of detecting carp down to a dilution equivalent to 9.3 kg carp/ha; however, the assay's detection success rate below the LOQ (160 kg carp/ha;~ 8 mg carp/L) was low. A lower LOQ could be obtained by either filtering a larger water volume, minimising the extraction volume from the filter, increasing the volume of extract in each PCR, or increasing the number of PCR reactions performed from each sample (Herder et al., 2014; Ficetola et al., 2015; Beng and Corlett, 2020). Such modifications could enhance eDNA's detection rates at lower biomass concentrations and thus its overall viability as an early detection method. However, these options may be impractical with natural samples. For instance, naturally sediment laden water samples often severely limit the volume of water that can be filtered (Herder et al., 2014). Supplementary to amplifying carp DNA, the "species specific" primer also amplified goldfish DNA, albeit with low levels of efficiency. This cross-species amplification suggests that the assay used in this study may need further optimisation before it is deployed as a carp detection method, as carp and goldfish commonly co-exist in natural aquatic systems and thus differentiation between the species is vital (Collier et al., 2015).

It has been demonstrated that scent-detection dogs can learn the position of a positive sample, and sometimes indicate a specific position rather than a specific sample (Johnen et al., 2015). While this could explain why the dog's performance on target and non-target samples improved in the second session, we believe this is highly unlikely given: (1) the large number of samples assessed by the dog (i.e., 17 samples per session); (2) the dog receiving no visual feedback on the position of the samples (as the segment positions were obscured by an opaque front panel); and (3) the 5-minute breaks between sessions. A more likely explanation for this sessional difference in detection performance is the accumulation of VOCs in the headspace of the segments over time. In enclosed spaces the concentration of VOCs increases until an equilibrium is met (i.e., no further increase in time will result in a greater concentration of the odour; Lazarowski et al., 2020). As equilibrium may not have been met after the 20-minute sit time, a lower concentration of VOCs may have been present in the first session, potentially making it harder for the dog to detect and differentiate carp from non-target scents. If dogs were to be deployed as a detection method, a timeframe in which the target samples are likely to meet headspace equilibrium should be determined to help maximise dogs' chances of a positive detection. Another possible explanation for the improved performance in the second session is that reinforcement of probe sample indications when the dog was first exposed to the lower concentration scents (in the first session) may have improved performance on their second exposure to these lower concentration scents (in the second session).

Theoretically, a dog could be taken to a waterbody to assess it for the presence of invasive carp, although this is likely to be time consuming and laborious. Laboratory-based assessment could provide a much more efficient method of invasive fish detection, as: (1) the dog would not need to be transported to and from site, reducing costs; (2) the dog could assess water samples from numerous lakes within a session; (3) the sample array could be assessed by more than one dog; and finally (4) stable sample characteristics and working conditions for the dogs could lead to improved detection performance.

Despite demonstrating that dogs can rapidly detect low carp biomass in laboratory-collected water samples, further research is warranted before dogs are deployed as a detection method for invasive carp. For example, this experiment should be repeated with a larger sample size (i.

e., more than one dog) as studies have demonstrated that dogs' motivation to work and olfactory acuity can vary across individual dogs and dog breeds (Jezierski et al., 2014; La Toya et al., 2017). Thus, these results may not be reflective of all dogs' detection sensitivity to carp scent. Dogs' responses to intermittent reinforcement on carp water samples also needs to be evaluated (Edwards et al., 2017). If water samples were to be sent into the laboratory for evaluation by dogs, the status of those samples would be unknown, thus reinforcement for a correct indication on those samples would not be possible. Finally, dogs' detection performance on water sourced from natural aquatic systems (e.g., lakes, rivers, ponds) should be assessed. Naturally sourced water is likely to contain significantly more distractor odours (i.e., VOCs released from aquatic vegetation, sediments, or fauna) than laboratory-collected water samples, potentially impacting the dogs' ability to detect carp.

5. Conclusion

In summary, scent-detection dogs may represent an accurate, efficient, and highly sensitive detection method for invasive carp. When presented water samples from laboratory aquaria, a detection dog could successfully detect and discriminate carp from two distractor scents, demonstrating similar sensitivity levels to eDNA. Use of scent-detection dogs could not only enhance invasive fish detection rates but could also facilitate more regular monitoring of lakes as once trained, dogs are likely to be more time, cost, and labour efficient than existing survey methods.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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