Canine Scent Detection: A New Approach to Detect Invasive Freshwater Fish

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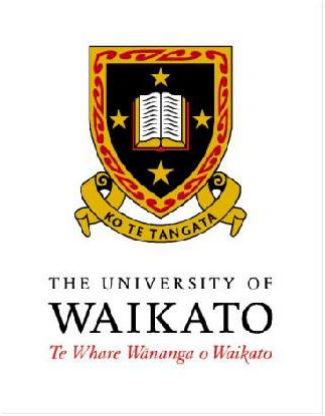
Melissa Anne Collins



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Abstract

Koi carp (*Cyprinus rubrofuscus*) were first introduced to New Zealand in the early 1960s. Since their introduction, they have contributed to the decline of New Zealand's freshwater ecosystems through resuspension of sediment from their feeding behaviour and through competition with native species. Methods such as netting, electrofishing, and environmental DNA (eDNA) are used to detect and monitor invasive fish species. However, these methods can be labour intensive, expensive, and can have low sensitivity when fish are rare or elusive. Although scent-detection dogs have been used to detect a wide range of substances, diseases, and animals, there is limited information on dogs' ability to detect aquatic species. This thesis aimed to determine if laboratory-based dogs could be used as a viable survey technique for invasive carp. Three studies were performed to investigate this aim.

The first study (Chapter 2) investigated dogs' sensitivity and specificity to carp scent using a multiple probe design experiment to determine if dogs had the potential to detect a low biomass of carp. A single dog was trained to use an automated carousel independently of its handler and assessed water samples from aquaria containing either no fish scent (non-target, n = 3), goldfish (*Carassius auratus*) scent (non-target, n = 5) or carp (target, n = 9) scent. 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 (equivalent to 310 kg fish/ha). The remaining three target samples (probes) were diluted to determine the dog's detection threshold. Results showed that the dog could detect carp down to a dilution equivalent to a biomass of 9.3 kg/ha (i.e., 0.5 mg carp/L), well below the biomass threshold of carp known to cause significant ecological impacts (i.e., >100 kg/ha). The dog's performance was then compared with eDNA, an existing survey method. Quantitative PCR conducted on DNA extracted from laboratory aquaria 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). Collectively, these results suggest that laboratory-based dogs could, with further research, provide a highly sensitive method of carp detection.

Depending on the location of the field site it could take hours or even days for water samples to reach the laboratory and be assessed by dogs. Without effective preservation the olfactory profile of the scent could change, making it unrecognisable to the dogs. While preservation of scent samples is commonly practiced in the literature, very few studies have investigated the impacts preservation methods can have on dogs' ability to detect the target scent. The second study of this thesis (Chapter 3) used an ABACADA reversal design to determine if refrigeration, freezing or potassium sorbate could be used as water sample preservation methods. To assess this, the dogs (n = 2-4) performed a baseline evaluation (A) of unpreserved water samples from aquaria containing carp (target scent; n = 7), goldfish (non-target scent; n = 5), or no fish (non-target scent; n = 5) before preservation treatments were applied to the samples. The treatment phases (B = refrigeration, C = freezing, and D = room temperature with potassium sorbate) involved applying a different preservation method to the water samples seven or eight days prior to assessment by the dogs. The results indicated that freezing and potassium sorbate have potential as scent sample preservatives. However, further research, testing longer storage durations with a larger sample size, is required to determine the full efficacy of these preservation techniques.

The final study in this thesis (Chapter 4) evaluated dogs' ability to detect carp in lake water. The first experiment in this study was a proof-of-concept experiment to determine if dogs could detect a common biomass of carp in lake water, which likely contains more background odours than aquaria water. Results from this experiment revealed that dogs could accurately detect a standard biomass of carp (i.e., 310 kg carp/ha) in samples from three lakes with varying water qualities; Taupō, Rotoroa and Rotoehu. All dogs achieved sensitivity

values >83.6% and specificity values >77.3% on all lakes tested. Experiment Two tested dogs' ability to generalise carp scent across lake samples that varied in novelty, trophic state, and carp biomass. The results from this experiment revealed that dogs' mean detection performance was above chance on some, but not all, lakes. To compare the dogs' detection performance to an existing survey technique, eDNA analysis was performed on water samples assessed by the dogs. Interestingly, the multispecies assay only detected carp in three out of five lakes. Quantitative PCR improved detection of carp but still failed to detect carp in a lake known to contain naturalised populations. These results indicated that dogs have the ability to detect carp in naturally sourced water. However, further research is necessary to determine the scope of their generalisation abilities given the limitations imposed by COVID-19 and their inconsistent performances during Experiment Two.

The findings from this thesis provide evidence that laboratory-based scent detection dogs may have potential to be used as an invasive carp detection method. In addition to demonstrating that dogs and eDNA have comparable levels of sensitivity to carp in aquaria water and that freezing, and potassium sorbate have potential as water sample preservatives, this thesis has also indicated that dogs may be able to detect and discriminate carp in water sourced from natural aquatic systems. The use of laboratory-based dogs could provide researchers with a timely and cost-efficient method of invasive fish detection, potentially enhancing their ability to regularly and extensively monitor freshwater ecosystems for new incursions of invasive species.

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Chapter 1

General Introduction

Harlee operating the automated scent detection apparatus.

1.1 Invasive Freshwater Species

Freshwater ecosystems are biodiversity hotspots covering <1% of the Earth's surface but contain approximately 6% of all taxonomically identified species (Dudgeon *et al.*, 2006; Balian *et al.*, 2008). They are integral to maintaining the earth's water cycle, and provide crucial ecosystem services such as food production, potable water, irrigation, filtration and nutrient cycling (Vanni, 2002; Dudgeon *et al.*, 2006). They also offer recreational activities (e.g., fishing, swimming, and boating) and have significant cultural and spiritual value for many communities (Millennium Ecosystem Assessment, 2005; Dudgeon *et al.*, 2006; Forslund *et al.*, 2009). Despite being an integral resource to all living organisms, human activities such as habitat destruction, pollution, over exploitation, and the introduction of invasive species threaten the integrity of freshwater ecosystems (Dudgeon *et al.*, 2006; Collier & Grainger, 2015).

The introduction of non-native species into freshwater ecosystems can have wide-ranging consequences (Dudgeon *et al.*, 2006; Strayer & Dudgeon, 2010; Francis & Chadwick, 2012). For hundreds of years, humans have deliberately (e.g., for fishing, aesthetic reasons, and pest control) or inadvertently (e.g., through animals escaping captivity or stowing away in transportation vehicles) introduced species into freshwater ecosystems. However, a rise in international trade and travel over the past century has caused an exponential increase in the rate of species introductions (Witmer *et al.*, 2007; Havel *et al.*, 2015; Muñoz-Mas *et al.*, 2023). Whilst most species introduced to an area outside their natural habitat do not establish a population, some become invasive and through competition, predation, hybridisation, or disease, cause significant harm to the environment and the economy (Witmer *et al.*, 2007; Havel *et al.*, 2015; Cuthbert *et al.*, 2021).

1.2 Koi Carp: A New Zealand Example

New Zealand has an abundance of freshwater habitats, including 413,000 km of streams and rivers, 3,800 lakes >1 ha and at least 249,214 ha of wetlands (Gluckman *et al.*, 2017; Dymond

et al., 2021). However, despite this abundance, the country has only 51 native fish species, 39 of which are either threatened or at risk of extinction (Stats NZ, 2020). In addition to habitat change, pollution and exploitation, a key factor contributing to the decline of New Zealand's freshwater fish are invasive species (Dean, 2001; Collier & Grainger, 2015). While New Zealand is recognised as a global leader in biosecurity (Meyerson & Reaser, 2002; Simberloff, 2014; Champion, 2018), 21 exotic fish species have been introduced to New Zealand since the 1840s (see Figure 1.1; Dean, 2001; Champion *et al.*, 2002; Collier & Grainger, 2015; Champion, 2018). Some of these species are considered low risk and are unlikely to spread beyond their current range, but other species, like koi carp (*Cyprinus rubrofuscus*) are considered high risk and have established self-sustaining populations in several freshwater habitats around New Zealand (Dean, 2001; Collier & Grainger, 2015).

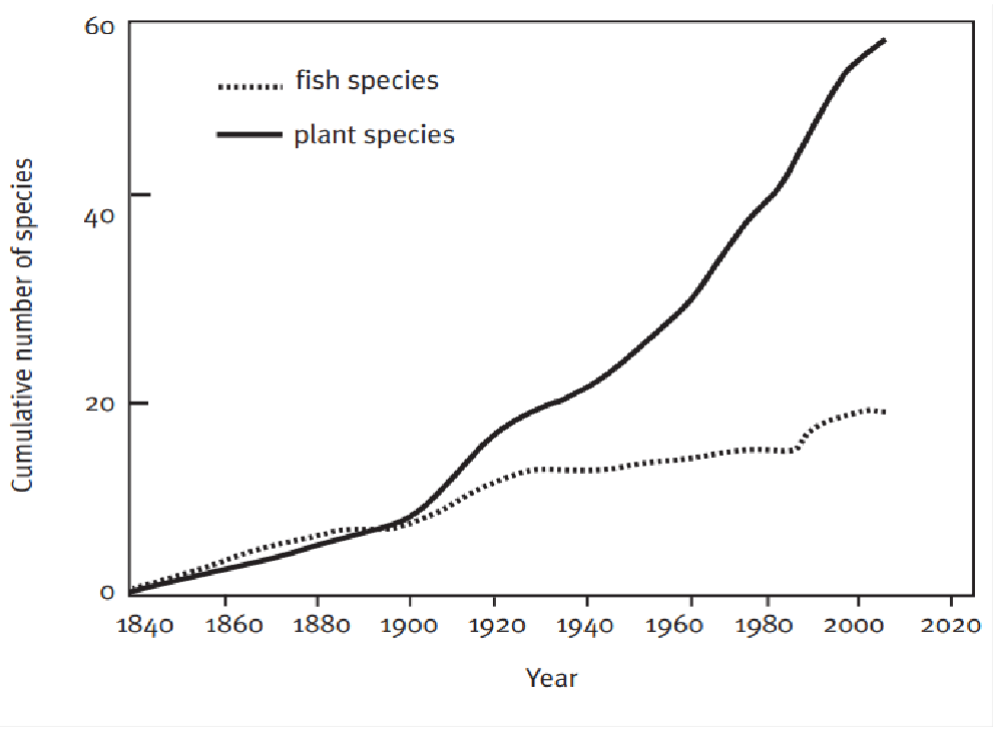

Figure 1.1: Dates of fish records for successfully established fish and aquatic plant species in New Zealand. Figure reprinted from: Lake Managers Handbook, Alien Invaders, by P. Champion., J. Clayton., & D. Rowe (2002). Prepared for the Ministry for the Environment, Wellington, New Zealand.

The origins of koi carp (*C. rubrofuscus*) in New Zealand are not clear, but it is believed that they were accidentally introduced with goldfish stocks in the early 1960s (Tempero, 2004). Shortly after their arrival, New Zealand officials allowed people who held a special permit to keep carp in captivity (McDowall, 1996). In the 1980s, a population of feral carp was discovered in the Whangamarino Wetland adjoining the Waikato River (McDowall, 1996). Carp control in this area presented challenges given the wetland's size (7,290 ha), diverse ownership and the potential impact of control measures on native biodiversity (McDowall, 1996). The government responded by creating a containment zone within the Auckland and Waikato regions, albeit this approach carried risks due to the presence of anglers who valued carp as a species for recreational fishing (McDowall, 1096). Due to carps' broad environmental tolerances, high fecundity, and human intervention, carp populations are now present across the North Island (see Figure 1.2), with reports of carp making up 90% of the fish biomass in some waterbodies within the Waikato Region (Osborne, 2006). Despite the carp in New Zealand belonging to the Asian linage of carp, and common carp (*Cyprinus carpio*) belonging to the European lineage, both species share similar biology and ecology, and henceforth these fish will be collectively referred to as carp.

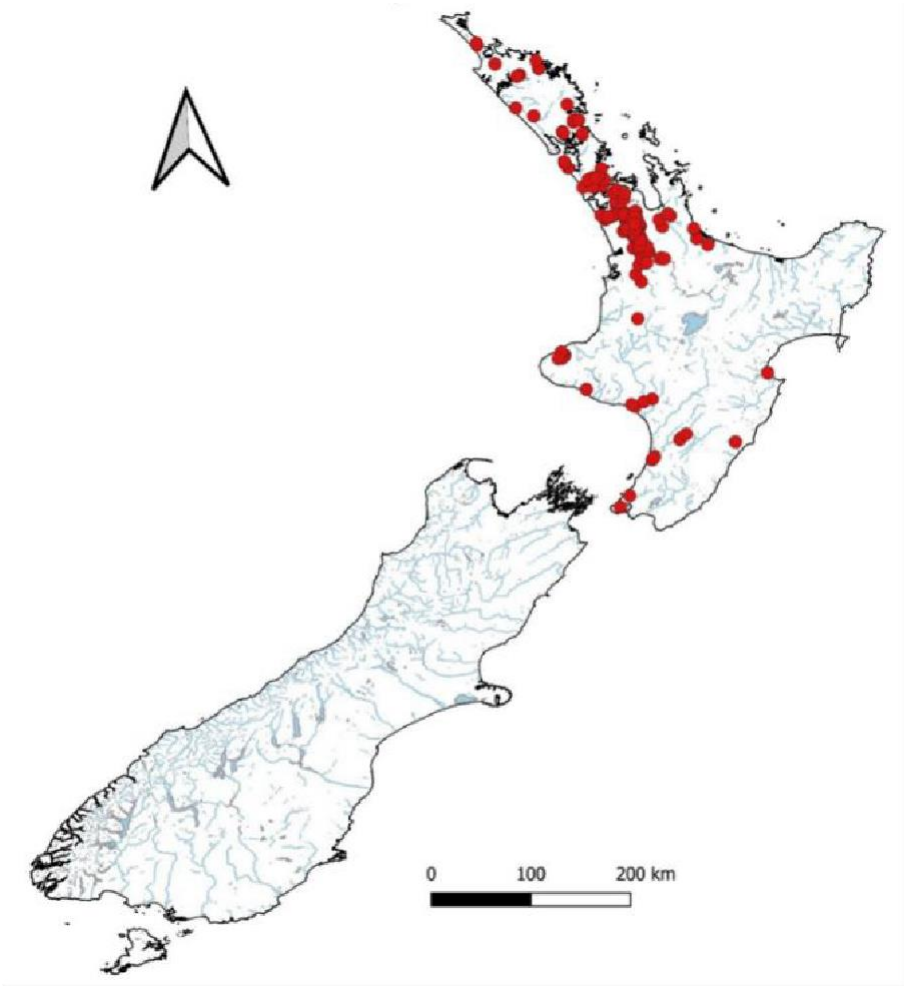

Figure 1.2: Known distribution of koi carp (*Cyprinus rubrofuscus*) in New Zealand. Figure reprinted from: New Zealand Freshwater Fish Database, December 2022.

1.3 Carp

1.3.1 Biology and Life History

Carp are found in freshwater ecosystems worldwide (Hicks *et al.*, 2011; Hicks & Ling, 2015). Wild carp generally live up to 4-5 years of age; nevertheless, there have been records of carp reaching ages of up to 34 years (Hicks & Ling 2015). Carp growth rates vary depending on the area and available resources. In general, male carp are smaller than females. For example, Tessema *et al.* (2020) revealed that the total length of females and males at first maturity was 21.5 and 17.5 cm, respectively, while Tempero *et al.* (2006) showed that females were significantly larger than males once they reached 6 years of age. Carp mature at a young age

and are highly fecund, allowing them to establish high-density populations quickly. In New Zealand, male carp typically mature at 2 years, whereas females typically mature at 3 years of age (Tempero *et al.,* 2006). The overall sex ratio of carp is normally weighted towards females, but it can vary depending on the location (Brown *et al.*, 2005; Joadder *et al.*, 2009). For example, Joadder *et al.* (2009) found that 52% of carp in Bangladesh were female, whereas Brown *et al.* (2005) discovered that only 30% of carp in Australia were female. Carp are prolific breeders, with a single female producing up to one million eggs per year (Swee & McCrimmon, 1966; Tempero, 2004; Brown *et al.*, 2005; Hicks & Ling, 2015). Once the eggs have been laid, they usually adhere to submerged vegetation and hatch when the conditions permit (Baldry, 2000; Korwin-Kossakowski, 2008). Typically, carp take less time to hatch at higher temperatures. For example, Korwin-Kossakowski (2008) demonstrated that it took 4.7 and 1.4 days on average to hatch when incubated at 20°C and 32°C, respectively.

Carp spawning patterns are strongly influenced by water temperature (Tempero *et al.*, 2006; Hicks & Ling, 2015; Watkinson *et al.*, 2021). Carp normally begin spawning when water temperatures range between 18°C and 28°C (McCrimmon, 1968), although spawning has been observed at 15°C (Stuart & Jones, 2002). During the spawning process, carp thrash about in shallow waters, resulting in the uplift of sediment and increased turbidity levels (Watkinson *et al.*, 2021). As carp spawning is temperature-dependent, research has shown that a single carp can spawn more than once a year if environmental conditions permit (Swee & McCrimmon, 1966; Tempero *et al.*, 2006).

Carp are benthic feeding omnivores and consume a wide range of food items including invertebrates, seeds, algae, detritus, and chironomids (Hicks & Ling, 2015; Huser & Bartels, 2015). As a result of their broad diet, carp can thrive in a range of environments and can outcompete native and other invasive species for resources (Hicks *et al.*, 2011; Hicks & Ling, 2015). Despite being used by humans for food, ornamental purposes, and recreational fishing,

carps' ability to tolerate a diverse range of environmental conditions and food sources, in addition to their high fecundity and rapid maturation, make them an extremely successful invasive species.

1.3.2 Ecological Impacts

Carp are well known for their destructive impact on the environment. When carp feed, they suck in nutrient-laden sediment and discharge indigestible material into the water column (Cahn, 1929; Roberts *et al.*, 1995; Zambrano & Hinojosa, 1999). This feeding behaviour, also referred to as 'mumbling', increases turbidity and nutrient levels within the waterbody (Roberts *et al.*, 1995; Hicks *et al.*, 2011; Hicks & Ling, 2015; Huser *et al.*, 2022). For example, Roberts *et al.* (1995) tested a range of carp densities (0–465 kg/ha) using 10 ponds within a commercial hatchery in the USA and revealed that as carp density increased, so did pond nutrient and turbidity levels. Moreover, it was discovered that this increase in turbidity adversely affected other biotic communities, including submerged macrophytes.

As carp increase turbidity levels, the amount of light that can penetrate the water surface decreases (Qiu *et al.*, 2019). This affects plant growth by decreasing their ability to photosynthesise (Qiu *et al.*, 2019). In addition to reduced light levels, carp may also reduce macrophyte abundance through direct consumption (Crivelli, 1981; Hinojosa-Garro & Zambrano, 2004), and physical up-rooting during feeding (Weber & Brown, 2009; Hicks *et al.*, 2011; Badiou & Goldsborough, 2015; Hicks & Ling, 2015). As bioturbators, carp can disturb the top 20 cm of lake sediments (Huser *et al.*, 2016; Qiu *et al.*, 2019). During this process, macrophytes are often dislodged from the sediment and left to decompose in the water column or on the lakebed. Given that macrophytes provide a refugia and food source for native species (Miller & Crowl, 2006), and play an important role in nutrient cycling, reductions in their populations can have significant adverse effects on ecosystem functioning.

Carp can also directly and indirectly affect the abundance of vertebrate and invertebrate species. Invertebrates are a common prey item for carp. As such, their populations are often reduced through direct consumption. For example, Hinojosa-Garro and Zambrano (2004) analysed the gut content of carp and found that they primarily ate invertebrates, detritus, plant tissues, and seeds. It has also been speculated that invertebrate populations are indirectly affected by loss of macrophyte abundance. Miller and Crowl (2006) investigated the effects of common carp on macrophyte and invertebrate communities in small and large enclosures in Utah Lake, United States. They discovered that the total benthic macroinvertebrate diversity decreased by 67% in the presence of carp relative to controls, and that this loss was strongly correlated with reductions in macrophyte abundance.

It has been suggested that carp directly reduce native fish populations by preying on their eggs and larvae; however, these prey items typically make up only a small proportion of carps' diet (Pietsch & Hirsch, 2015). Instead, the effects of carp on native fish diversity are primarily indirect. As a highly fecund species, which have a rapid growth rate and a broad diet, carp often outcompete native fish for food resources and habitat, resulting in their decline or, in the worst case, extinction. Carp also affect the population and diversity of native fish through sediment resuspension (Wood & Armitage, 1997). When sediment is resuspended in a water column, it can clog the gills of native fish that are not equipped to live in highly turbid water (Wood & Armitage, 1997). Moreover, increased sediment levels can impact the suitability of spawning habitats and impede the development of larvae and juvenile fish, which are more susceptible to high sediment levels than their adult counterparts (Wood & Armitage, 1997).

In addition to invertebrates and fish, carp have been strongly implicated in the reduction of waterfowl and wetland bird populations (Badiou *et al.*, 2011). Waterfowl and other avian species depend on chironomids and pond weeds for their food (Badiou *et al.*, 2011). Carp commonly consume these items, reducing the amount of food available for these bird species

(Badiou *et al.*, 2011). At a site near München, Germany, it was discovered that bird populations were much smaller (~10 waterbirds/ha) in ponds stocked with 500 kg carp/ha, relative to bird populations found in ponds that were carp-free (300 waterbirds/ha) (Köhler *et al.*, 1997; Badiou *et al.*, 2011). A more recent study at the same site revealed that benthivore fish and water birds compete for food resources and that the effects of this competition are dictated by the density of fish (Haas *et al.*, 2007).

1.3.3 Biomass Impact Threshold

Carp can cause ecosystem regime shifts, turning shallow lakes from a clear macrophyte-dominant state to a turbid, phytoplankton-dominant state (Hicks *et al.*, 2011; Vilizzi *et al.*, 2014; Hicks & Ling, 2015). However, these regime shifts occur only when carp biomass reaches a critical threshold, which can vary depending on the water body (Hicks *et al.*, 2011; Vilizzi *et al.*, 2014). For example, Vilizzi *et al.* (2014) conducted a 2-year experiment in a semi-arid region in south-eastern Australia and discovered that in addition to a major decrease in invertebrate richness, water quality also deteriorated and became highly turbid at a carp biomass of 68.0 kg carp/ha. In contrast, Bajer *et al.* (2009) conducted a multi-year study in a lake in the mid-western USA and revealed that a significant decline in vegetation and waterfowl abundance occurred at ~100 kg carp/ha. Given that carp typically cause extensive damage to freshwater ecosystems at biomasses that range between 68–450 kg/ha (Fletcher *et al.*, 1985; Williams *et al.*, 2002; Haas *et al.*, 2007; Matsuzaki *et al.*, 2009; Vilizzi *et al.*, 2014; Collier & Grainger, 2015; Hicks & Ling, 2015), it is important to detect invasions early when their densities are low, and to implement rapid control actions.

1.4 Current Fish Detection Methods

Currently, four primary methods are used to detect carp and other fish species in freshwater ecosystems, these being: visual observations, electrofishing, netting, and environmental DNA (eDNA).

1.4.1 Visual Observation

Visual searches have been used to detect and monitor invasive fish species in ponds, rivers, and lakes. To conduct visual searches, the observer usually wears polarised glasses to reduce light reflection from the water and allow better visibility of the fish (Grainger & McCaughan, 2014). Although this method is fairly simple to perform (i.e., marking the presence, absence, or location of a fish species), it does require someone with taxonomic expertise to ensure that taxa are identified correctly, and can only be performed in shallow water (Grainger & McCaughan, 2014). In New Zealand, carp and goldfish are morphologically similar, albeit carp can be identified by their 'barbles' which are whisker-like feeders on the side of their mouth. Often when performing visual searches such characteristics are too small to see at a distance. Carp also reduce water clarity when they feed (Roberts *et al.*, 1995) and as such visual observations can become difficult and false negatives can occur (i.e., failure to detect the target organism when it is present in the waterbody). Thus, when surveying a water body for carp, visual observations may be best performed in conjunction with other methods such as electrofishing, netting, or eDNA.

1.4.2 Netting

Set nets, where fish encounter and enter the net, are frequently deployed in lakes and rivers to capture both invasive and native fish species (Murphy & Willis, 1996). Netting surveys are generally considered a relatively low-tech and cost-effective method for fish capture. Three

main types of set nets are used to capture carp in New Zealand: gill, trammel, and fyke nets. Gill and trammel nets typically consist of 1 or 3 layers of mesh, respectively, and are deployed vertically in the water column, whereas fyke nets are a cylindrical fish trap which has a series of funnel shaped openings (Murphy & Willis, 1996; Lake, 2013ab). Despite slight differences in structure, these nets all have similar limitations: (1) they can be labour intensive when fish densities are low and require frequent checking and removal of by-catch (Lake, 2013 ab; Collier & Grainger, 2015); (2) they can catch, injure, or kill non-target species (Lake, 2013ab; Collier & Grainger, 2015); (3) they can be size-selective depending on the mesh sizes deployed to capture fish (Lake, 2013ab); and finally (4) predation can occur within the net, impacting the accuracy of capture rates (Murphy & Willis, 1996; Lake, 2013ab). Studies have also demonstrated that although these netting methods can capture carp, other methods such as electrofishing are more effective, particularly in shallow waters. For example, Hicks *et al.* (2015b) revealed that fyke nets were 21–52% efficient for catfish (*Ameiurus nebulosus*) and eels (*Anguilla* spp.), but only 2% efficient for goldfish and caught no koi carp. In contrast, boat electrofishing was 13–22% efficient for goldfish and carp, but only 2–6% efficient for catfish and eels.

1.4.3 Electrofishing

Electrofishing is one of the main methods used to monitor native and invasive fish in New Zealand waters (Collier & Grainger, 2015). It involves the use of electricity to capture fish. Two electrodes, an anode and cathode, are submerged in the water and electrical pulses are released. These pulses cause involuntary muscular contractions in the fish, resulting in the fish swimming toward the electrical stimulus. Once the fish is close to the electrodes, the electrical pulses induce narcosis (a sleep-like state in the fish) allowing the fish to be easily captured (Sharber *et al*., 1994). There are three types of electrofishing techniques: backpack, bank-

mounted, and boat mounted (Portt *et al*., 2006). Backpack and bank-mounted electrofishing are typically used to capture fish in small wadable streams or rivers, while boat electrofishing is used for sampling lakes and large rivers (Portt *et al*., 2006; Collier & Grainger, 2015). Electrofishing is most effective for capturing carp in shallow waters without much habitat complexity. In deep water with diverse habitats, the electrical field is often reduced or impaired, affecting its overall efficacy as a detection method (Portt *et al.*, 2006; Collier & Grainger, 2015; Kusabs *et al*., 2018).

Despite being one of the most effective detection methods, the noise generated by the boat-mounted generators can be loud, potentially scaring the aquatic species before they enter the range of the electrodes (Collier & Grainger, 2015; Kusabs *et al.*, 2018). Electrofishing may also cause physical injury to fish if the amplitude and frequency of the electrical pulses are not properly regulated (Sharber *et al.*, 1994; De Villiers, 2013; Hicks *et al.*, 2015a). For example, Reynolds and Holliman (2004) conducted a study in the St Lawrence River USA and found that electrofishing at 30 Hz pulsed DC (PDC) with a peak voltage of 336 V and a peak current of 4–5 amps resulted in spinal damage to 60% of the 18 American eels (*Anguilla rostrata*) captured and caused haemorrhages in 30% of the fish caught. Similarly, Hicks *et al.* (2015a) performed PDC boat electrofishing in the Waikato River, New Zealand (PDC = 60 Hz; 50-500 V) and discovered that 14% of the 29 grey mullet (*Mugil cephalis*) and 10% of the 1,278 smelt (*Retropinna retropinna*) experienced spinal injury. To avoid harm or physical injury to a species, non-invasive methods (i.e., cause no harm the target species), such as environmental DNA (eDNA), can be used (Rees *et al.*, 2014).

1.4.4 Environmental DNA

Environmental DNA is a method of detecting the presence of a species from the cells they shed into the environment (Takahara *et al.*, 2013; Keskin, 2014; Rees *et al.*, 2014). Once the sample

has been collected from the environment and brought back to the laboratory, the DNA is isolated from the sample and amplified by polymerase chain reaction (PCR) or quantitative PCR (qPCR) (Rees *et al.*, 2014; Furlan *et al.*, 2016). DNA amplification[1] only occurs if the target DNA sequence matches with the gene template of the PCR primer. If they do not match, no copies of the DNA are made. Amplified DNA sequences are then analysed using high-throughput sequencing platforms and cross-referenced with genetic databases to identify the species present (Melchior & Baker, 2023).

Environmental DNA offers advantages over traditional survey methods, such as netting and electrofishing, because it can be highly sensitive (i.e., it can detect species at low densities; Takahara *et al.*, 2013; Rees *et al.*, 2014), it is non-invasive, and can be relatively cost-effective once the technique has been optimised (Jerde *et al.*, 2011; Sigsgaard *et al.*, 2015; Smart *et al.*, 2016; Evans *et al.*, 2017). It is also useful in situations where species are morphologically similar and difficult to differentiate without taxonomic expertise (Furlan *et al.*, 2016).

Despite these advantages, eDNA has several limitations that can affect its use and accuracy as an invasive fish detection method (Strickler *et al.*, 2015). The rate at which a species sheds DNA into water and the patchiness of its distribution can dictate detection rates (Strickler *et al.*, 2015; Furlan *et al.*, 2016). If a species is present at high densities, DNA is likely to be evenly distributed throughout the water body, and there is a greater chance of detection (Strickler *et al.*, 2015; Furlan *et al.*, 2016). Comparatively, if the species is present at low densities or is spatially clumped, then the probability of detecting the target organism's DNA is reduced.

Supplementary to the spatial distribution and abundance of species in a water body, recent research has demonstrated that DNA can quickly degrade in water. Laboratory

[1] DNA amplification can be defined as the process of creating copies of a particular region of DNA (the amplicon), usually through a PCR reaction using primers and enzymes such as polymerases (Melchior & Baker, 2023).

experiments have revealed that New Zealand mud snail (*Potamopyrgus antipodarum*) and American bullfrog (*Lithobates catesbeianus*) tadpole DNA persisted for only 21 and 25 days, respectively, following species removal (Dejean *et al.*, 2011; Goldberg *et al.*, 2013). Similar rates of DNA degradation have also been reported in other experiments: DNA from two larval amphibian species persisted in water for 7–14 days following their removal (Thomsen *et al.*, 2012), whereas Burmese python (*Python bivittatus*) DNA persisted in water for 2–7 days (Piaggio *et al.*, 2014). Despite these studies demonstrating the rapid degradation of DNA in water, it is important to consider environmental factors (e.g., temperature, UV, pH, hydrological regime) and how they can influence DNA persistence.

Pilliod *et al.* (2014) used outdoor containers to examine the impact different light and temperature regimes had on the persistence of salamander (*Dicamptodon aterrimus*) DNA in water. The authors identified three key findings: (1) under ambient temperature and light, eDNA was detectable for up to 8 days; (2) under ambient temperature and reduced light, eDNA was detectable for at least 11 days; and (3) eDNA was detectable for at least 18 days under refrigerated (4°C) conditions with no light. A microcosm study conducted by Strickler *et al.* (2015) also revealed that under low ultraviolet (UV), cold temperatures (5°C), and alkaline water conditions, American bullfrog DNA degradation rates were lower than under high UV and high temperatures conditions. The authors attributed this result to the latter conditions being more favourable for microbes to break down their DNA.

The hydrological regime of a water body can also influence the rate of eDNA degradation. In a study investigating the persistence of European weather loach (*Misgurnus fossilis*) eDNA in lotic (fast-flowing water bodies, e.g., rivers) and lentic (stagnant water bodies, such as lakes or ponds) systems, the probability of detecting weather loach eDNA was lower in lotic systems than in lentic systems (Thomsen *et al.*, 2012). Balasingham *et al.* (2017) reported similar results after releasing Atlantic salmon (*Salmo salar*) into a fast-flowing,

uncontrolled river located in southwestern Ontario, Canada. The authors reported that eDNA was only detectable at the release site for up to 11.5 hours. Moreover, significantly lower levels of eDNA were found slightly downstream of the source site, which the authors attributed to substrate settling, dilution, and degradation during suspension and transport.

Contamination is a major factor limiting the effectiveness of eDNA and may occur at any stage of the eDNA sampling, extraction, and amplification processes. For example, a mesocosm study conducted by Kelly *et al.* (2014) revealed that approximately a quarter of the DNA sequenced from their tanks originated from species that were not present in their experiment. Most of the DNA came from humans (62%), while a smaller proportion (29.2%) of the non-tank species DNA came from other vertebrate species (cow, *Bos taurus*; pigs, *Sus scrofa domesticus*; chicken, *Gallus gallus domesticus*; turkey, *Meleagris gallopavo*; and sea otters, *Enhydra lutris*). It was suggested that some or most of this exogenous DNA came from fish food or from intake water, which was drawn from a natural waterbody. Stoeckle *et al.* (2017) also showed that false positives (i.e., indication of the presence of a species when they are absent from a water body) can occur during eDNA analysis. The study suggested that in approximately 10% of the water samples collected from the lower Hudson River estuary (USA), DNA from rare or locally absent species (Atlantic salmon, Pacific red snapper, *Lutjanus peru*, European sea bass, *Dicentrarchus labrax*) was detected. As the presence of these species was highly unlikely, the authors concluded that their DNA likely originated from wastewater that flows directly into the estuary or from contamination during laboratory procedures. In addition to contamination, cross-species amplification can result from insufficient primer optimisation during the primer development process, a process that can be time consuming and difficult to conduct (Goldberg *et al.*, 2016; Collins *et al.*, 2022).

Detection errors, in particular false positives, are a concern for those investigating the persistence of eDNA in a water body, as they can lead to false assumptions or the

implementation of unnecessary actions (e.g., deployment of control programs) which can cost money or further impact non-target fish species. As a non-invasive detection method, environmental DNA can only provide information on the presence or absence of a species (Rees *et al.,* 2014). Therefore, if information about the life stage, reproduction, or fitness of a species is required, capture-based techniques need to be employed. A final limitation of eDNA is its limited ability to detect hybrid species (Rees *et al.,* 2014). Given its high abundance per cell, most eDNA assays are designed to target the species' mitochondrial DNA (mtDNA). However, mtDNA is maternally inherited and thus excludes information on the paternal linage, making it difficult to determine whether the species is a hybrid (Rees *et al.,* 2014). This could be problematic in New Zealand, as carp and goldfish naturally hybridise and can create fertile offspring (Tempero, 2004; Smith & McVeagh, 2005). Environmental DNA can be rapid, cost effective, and highly sensitive relative to capture-based techniques. However, like all other detection methods, eDNA has several limitations that can hinder its detection success and reliability as a surveillance method. Investigations into other potentially more efficient fish detection methods are therefore required.

1.5 Scent Detection Dogs: Are they the answer?

1.5.1 Canine Olfactory System

Olfaction is the main sense used by dogs. They use it to locate or select prey, for recognition of conspecifics or predators as well as for orientation and communication (Jendrny *et al.*, 2021). Consequently, dogs have developed a highly attuned olfactory system that is 10,000 to 100,000 times more sensitive than humans (Walker *et al.,* 2003; Walker *et al.,* 2006). Odour detection in dogs is a multi-step process. When a dog inhales, air enters the nostrils via two pathways: the upper flow path, which directs 12–13% of each breath to the olfactory region, and the lower flow path, which directs the remainder of the air to the lungs (Kokocińska-Kusiak *et al.*, 2021). Once air reaches the olfactory region, it passes over the olfactory epithelium, which comprises

of millions of bipolar neurons, also referred to as olfactory receptor cells (ORC) (Kokocińska-Kusiak *et al.*, 2021). These ORC have cilia on their surfaces that are covered with mucus. Odorants that vaporise at room temperature (hereafter referred to as volatile organic compounds or VOCs) diffuse through the mucus and attach to odour-specific olfactory receptors (Kokocińska-Kusiak *et al.*, 2021). Once attached to the ORC, electrical impulses are generated, sending information to the olfactory bulb (OB), which plays a modulatory and sensory role. The OB processes and filters information received from the ORC. From the OB, signals are sent to the olfactory cortex, which comprises of four components: the anterior olfactory nucleus, piriform cortex, peri-amygdaloid cortex, and the entorhinal cortex. The anterior olfactory nucleus, piriform cortex, and peri-amygdaloid cortex send signals to the frontal cortex and thalamus, which then transmit electrical pulses to the hippocampal formation, a structure involved in memory recognition of odours (Kokocińska-Kusiak *et al.*, 2021).

Research has shown that dogs can detect VOCs at concentrations as low as 1-2 parts per trillion (Walker *et al*., 2006). This sensitivity is far superior to humans and most other mammals. The reason for dogs' superior sense of smell is not fully understood but is thought to be related to the size and number of specific structures within their olfactory system. For example, Kavoi & Jameela (2011) conducted a morphometric analysis of the OB of dogs, humans and goats. The results from this experiment revealed that the volume of the olfactory bulb was greatest in dogs, followed by goats, and humans, constituting 0.31%, 0.18%, and 0.01%, respectively, of the brain volume. Other research has also demonstrated that dogs' olfactory epithelium is 30-fold larger and contains 3-fold more ORC than humans (Lippi & Heaney, 2020). Although more research is required, these factors likely contribute to dogs' superior olfactory abilities.

1.5.2 Conservation Detection Dogs

Owing to their acute sense of smell and trainability, humans have used dogs for a wide range of scent detection tasks (Browne *et al.*, 2006). For example, dogs have been used by medical researchers to detect diseases, law enforcement agencies to detect explosives, land mines, illicit substances, accelerants, and missing persons, and conservationists to detect plant and animal species (Browne *et al.*, 2006).

Dogs were first used for conservation purposes in the late 1880s when Richard Henry trained dogs to locate kiwi (*Apteryx* spp.) and kakapo (*Strigops habroptilus*) for translocation to a predator-free island (Hill & Hill, 1987; Browne, 2005; Beebe *et al.*, 2016). Since then, dogs have been deployed worldwide and have detected a plethora of native or invasive organisms. For example, dogs have detected brown tree snakes (*Boiga irregularis*; Engeman *et al.*, 2002), spotted knapweed (*Centaurea stoebe*; Goodwin *et al.*, 2010), red imported fire ants (*Solenopsis invicta*; Lin *et al.*, 2011), Norway rats (*Rattus norvegicus*; Shapira *et al.*, 2011), lizards (*Naultinus manukanus*; Browne *et al.*, 2015b), and frogs (*Pyxicephalus adspersus*; Matthew *et al.*, 2021).

Comparisons between scent detection dogs and existing survey techniques have revealed that dogs are often more efficient in detecting species, particularly at low densities (Homan *et al.*, 2001; Smith *et al.*, 2001; Grimm-Seyfarth *et al.*, 2019; Grimm-Seyfarth *et al.*, 2021). In Vermont, USA, the detection performance of dogs trained to detect the scats of black bear (*Ursus americanus*), fishers (*Martes pennanti*), and bobcats (*Lynx rufus*) was compared with the detection performance of hair snares and camera traps, which are commonly used to detect these elusive species. The results from this study revealed that dogs detected between 3.5–4.7 times more black bears, fishers, and bobcats than camera traps, and seven times more black bears than hair snares, which failed to detect any other target organism (Long *et al.*, 2007; Grimm-Seyfarth *et al.*, 2021). In Germany, a study compared dogs' detection of Eurasian otter

(*Lutra lutra*) scat to human surveyors (Grimm-Seyfarth *et al.*, 2019). They found that dogs not only detected four times more scat than human surveyors, but also conducted their surveys in significantly less time (Grimm-Seyfarth *et al.*, 2019). Similar results were found by Smith *et al.* (2001), where dogs detected four times more San Joaquin kit fox (*Vulpes macrotis mutica*) scats along a transect than an experienced scat detection person. Collectively, these studies demonstrate that dogs are a highly valued asset for terrestrial species detection, often outperforming existing survey techniques.

1.5.3 Scent Detection of Aquatic Species

In addition to terrestrial species, dogs have proven to be effective in the detection of aquatic organisms. Rolland *et al.* (2007) trained dogs to detect North Atlantic right whale (*Eubalaena gacialis*) scat to determine whether they could outperform human surveyors. When the detection performance of both survey methods was compared, dogs situated at the bow of the boat detected four times more North Atlantic right whale scats than humans conducting opportunistic surveys (Rolland *et al.*, 2007). Given the success of dogs in this study, they have since been deployed to detect not only North Atlantic right whales (Rolland *et al.*, 2017) but also orca (*Orcinus orca*) scats (Wasser *et al.*, 2017).

Dogs have also been used to detect invasive quagga mussels (*Dreissena rostriformis bugensis*) in both field and laboratory settings (DeShon *et al.*, 2016; Sawchuk, 2018). In America, dogs have been used as a screening method, where watercraft are inspected for the presence of mussels before they enter the water (Sawchuk, 2018). Some areas of the United States have even developed a system in which boats that have passed an inspection by a mussel detection dog are given a coloured sticker, which expedites their launching process when displayed to the harbour master (DeBruyckere *et al.*, 2018). Given that mussel larvae are not visible to the naked eye, DeShon *et al.* (2016) conducted a laboratory-based study to determine

whether dogs could detect larvae from water samples using a scent line up. Although the experimental design of this study could have been improved by ensuring immediate reinforcement for positive samples and increasing the number of samples assessed per trial, the study revealed that dogs could not only detect quagga mussel larvae in water samples, but they could do so at concentrations similar to those found in the field.

1.5.4 Challenges with Scent Detection Research

Scent detection can take place in the laboratory or in the field where the dog detects the organism or substance in-situ. In both instances, the dog is normally accompanied by a human handler who records its detection responses, provides reinforcement, and monitors its behaviour and well-being during a trial or a search. While handlers are often necessary, they can give unintentional cues to the dog which can impact the validity of the detection results. For example, Lit *et al.* (2011) discovered that dogs were more likely to indicate on a non-target sample when their handlers were falsely informed of the location of the target scent, suggesting that dog detection performance was being influenced by the handlers' actions (e.g., posture, eye contact, hand signals). Moreover, Zubedat *et al.* (2014) revealed that the performance of dogs increased when their handlers were stressed. This increase in detection performance was attributed to handlers paying less attention to dogs, thus reducing the number of cues given (Johnen *et al.*, 2017; Lazarowski *et al.*, 2020). Blind trials, where the handler (i.e., single-blind), or the handler and the researcher (i.e., double-blind) are not aware of the position of the target have been used to minimise the impact of unintentional cueing. However, even under these conditions, the presence of a handler could still result in unintentional cuing. Moreover, blind trials can create problems with the timing of reinforcement. When the handler does not know the position of the target sample, immediate reinforcement for a correct indication is seldom

possible. As demonstrated by Browne (2015a), delayed reinforcement can affect a dog's ability to learn a task, as well as their motivation to work.

Recent studies have explored the use of automated scent detection apparatuses, in which dogs are trained to assess samples independently of their handlers (Edwards, 2019; Aviles-Rosa *et al.*, 2021). While these devices negate the issues associated with handler cueing and can provide immediate reinforcement for correct indications, they can be expensive to build and are only suitable for laboratory-based scent detection (Edwards, 2019; Aviles-Rosa *et al.*, 2021).

Dogs are complex biological organisms; they are not neatly constructed mechanical devices. Consequently, their detection performance can be impacted by a number of variables. For example, research has shown that dogs are 1.4 and 1.5 times more likely to detect explosive material before exercise or when they are on a high polyunsaturated diet, respectively (Angle *et al.*, 2014). It has also been demonstrated that medications (Jenkins *et al.*, 2016), diseases (Myers *et al.*, 1988), excessive training (Demant *et al.*, 2011), handler experience (Hayes *et al.*, 2018) and the dogs' working and living conditions (i.e., temperature and humidity; Savidge *et al.*, 2011; Chambers *et al.*, 2015; Troisi *et al.*, 2019) can have an adverse impact on the olfactory capabilities of dogs and their motivation to work.

Dogs often enhance our ability to detect a range of substances and organisms, including illicit drugs, explosives, threatened or invasive species, and diseases (e.g., cancer). However, a common challenge with scent detection dogs is that not all complete their training, and many dogs are withdrawn prematurely from service due to behavioural or accuracy issues (Troisi *et al.*, 2019; Cobb *et al.*, 2021). Such withdrawals can be problematic as they can cost researchers' significant amounts of time and money.

The expense of developing and appropriately caring for scent detection dogs can make them an unrealistic option for various groups or organisations (Arandjelovic *et al.*, 2015; Chambers *et al.*, 2015; Rutter, 2021). To reduce costs and improve accessibility, recent

literature has investigated the potential use of pet dogs as scent detectors, as: (1) they do not require permanent housing at the scent detection facility, and (2) the owner is responsible for the dog's veterinary, maintenance and food costs (Rutter, 2021). While pet dogs have proven efficacious in several scent detection tasks, there are limitations associated with their use. Firstly, dog attendance is dependent on the owner schedule, potentially restricting the researcher's ability to conduct trials. Also, the researcher has little control over the dogs' diet or exercise regime; factors which can impact dogs' detection performance (Gazit & Terkel, 2003; Miller & Bender, 2012).

Laboratory-based scent detection can provide certain advantages over field surveys. For example, researchers have better control over environmental factors (i.e., temperature, humidity, wind, rain) that are known to impact dogs' olfactory capabilities (Reed *et al.*, 2011; Savidge *et al.*, 2011; Chambers *et al.*, 2015). A sample array set up for one dog can be assessed by multiple dogs, improving experimental validity (Edwards, 2019). Dog-handler duos do not need to be transported to and from the field site, potentially improving time and cost efficiency. Finally, dogs can be trained to assess samples in the absence of a handler, negating issues associated with handler cueing (Edwards, 2019). Despite these advantages, there are disadvantages associated with laboratory-based scent detection. One significant limitation is the need to collect samples from the field or the target environment and transport them back to the laboratory, where they are assessed by the dog. The dynamic nature of scent profiles means that delays in assessment can result in changes to the scent that may render it unrecognisable to the dogs (Forbes *et al*., 2014). To minimise this issue, researchers often apply cold storage methods, such as refrigeration or freezing to the sample. Despite having proven effective in delaying scent degradation in many instances, more research is required to determine how long these preservation methods can maintain a scent's original olfactory profile, and if this duration changes with sample type (Matthew & Relton, 2021; Needs *et al.*, 2021).

In summary, dogs have a highly developed olfactory system with sensitivity many orders of magnitude greater than humans, making them valuable tools for conservationists. In addition to detecting terrestrial and aquatic organisms, dogs have also outperformed existing survey techniques on several occasions. Despite being a highly sensitive and efficient detection method, there are factors that can limit the use of scent detection dogs (i.e., expense) or detection success (i.e., handler cueing, diet, exercise regime, disease, medication). Before deploying dogs as a detection technique, it is essential to consider these limitations and develop a methodology that minimises their impact on the dog's detection performance. For example, pet dogs could be trained to evaluate water samples using a fully automated scent detection apparatus. This approach would be more cost effective than using dogs permanently housed at the scent detection facility. Moreover, the use of an automated apparatus would negate issues associated with human cueing and subjectivity.

1.6 Thesis Outline

The invasion of koi carp into New Zealand's freshwater ecosystems poses a significant threat to native biodiversity. Because of the limitations associated with current detection techniques, a more effective method of pest fish detection is required. Given dogs' success in detecting rare and elusive species in both terrestrial and aquatic environments, this thesis aimed to determine if laboratory-based dogs trained to operate an automated scent detection apparatus could be used to detect invasive carp from water samples. To evaluate this matter, three research questions were posed.

Chapter 2: Are dogs capable of detecting carp at biomasses similar to what would be present when a new invasion occurred? Moreover, how does their detection performance compare to environmental DNA, an existing survey method?

- This chapter investigated the sensitivity and specificity of dogs to carp scent in aquarium water and compared their detection performance to environmental DNA. This

study was conducted to determine: (1) if dogs could be utilised as an early detection tool and detect carp before they reach ecologically harmful densities (<100 kg carp/ha), and (2) how the dog's sensitivity compared to eDNA.

Chapter 3: Can dogs detect the presence of carp in preserved water samples?

- If water samples are to be sent to the laboratory for evaluation by dogs, it could take hours or days for the sample to reach the laboratory. Without effective preservation, the original olfactory profile of a scent could change, potentially making it unrecognisable by dogs. Chapter 3 investigated dogs' ability to detect carp in water that had been stored for 7 or 8 days at either 4°C, -18°C, or at room temperature with a set concentration of potassium sorbate to determine which was the most effective preservation technique for canine-assessed water samples.

Chapter 4: Can dogs detect carp in naturally sourced water?

- To be an effective detection technique, dogs need to be able to detect carp in naturally sourced water, which likely contains more non-target odours than aquarium water. Chapter 4 evaluated the ability of dogs to detect and generalise carp scent in water collected from lakes within the Waikato and Bay of Plenty regions of New Zealand.

In the final section of this thesis, a general discussion is presented (Chapter 5). This discussion summarises the main findings of the study, explores their implications, and acknowledges the limitations encountered during the research process. Moreover, future recommendations are discussed to provide directions for future research and potential actions based on the studies' outcomes.

1.6.1 Publication Status of Research Chapters

Of the five thesis chapters, three were prepared as manuscripts for publication in scientific journals. Because of this, there will be some repetition of topic introductions, methodologies, and themes or concepts across chapters. The current publication status of these chapters is outlined below.

Chapter 2 was published as follows: Collins, M. A., Browne, C. M., Edwards, T. L., Ling, N., Tempero, G. W., Gleeson, D. M., Crockett, K., & Quaife, J. (2022). How low can they go: A comparison between dog (*Canis familiaris*) and environmental DNA detection of invasive koi carp (*Cyprinus rubrofuscus*). *Applied Animal Behaviour Science, 255,* 105729. https://doi.org/10.1016/j.applanim.2022.105729.

- Chapter 3 is in preparation for submission to the Journal of Applied Animal Behaviour Science.
- Chapter 4 is in preparation for submission to the New Zealand Journal of Zoology.

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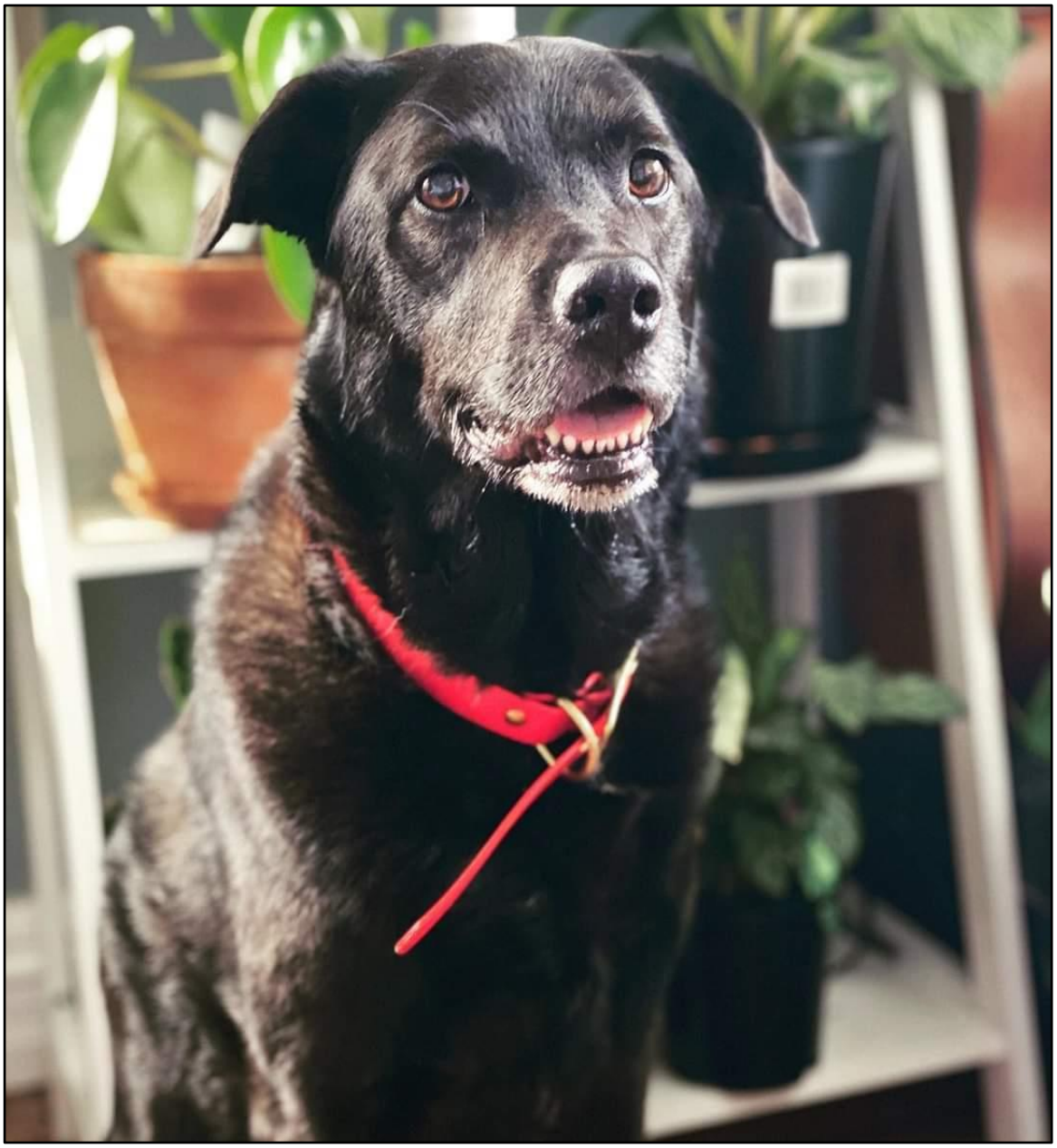
Chapter 2

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



Scent detector dog Ruby. Photo credit: Amy Barker.

Why determine dogs' sensitivity to carp, and compare their detection performance to eDNA?

The optimum goal for aquatic conservationists and/or fishery managers is to prevent invasive species from entering a new area or waterbody. However, some species will slip through prevention efforts and establish low density populations (Lodge *et al*., 2006[2]). Depending on the species, there is typically a time lag between establishment and spread (Lodge *et al*., 2006). Detecting a species before it spreads increases the likelihood of eradication and reduces the chance of that species causing irreversible ecological or economic damage (Lodge *et al*., 2006). In the case of carp, obvious changes in water quality and ecosystem health typically occur when carp exceed a biomass of 100 kg/ha (King *et al.*, 1997[3]; Chumchal *et al.*, 2005[4]; Driver, 2005[5]; Bajer *et al.*, 2009[6]). Current methods used to detect carp are either time-consuming, costly, or lack sensitivity at low fish densities. Given dogs' acute sense of smell and success at detecting rare and elusive species, they could be used as an early detection method for carp. To determine the potential utility of dogs as an early detection method, Chapter 2 evaluated a dog's sensitivity to carp scented water samples collected from laboratory aquaria. It also compared the dog's detection performance to eDNA to determine if dogs are more efficient at detecting carp than an existing surveillance technique.

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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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Author contributions:

Conceptualisation: Melissa Collins (MC), Clare Browne (CB), Timothy Edwards (TE), Nicholas Ling (NL), Grant Tempero (GT), Dianne Gleeson (DG); Experimental Design: MC, Kymberly Crocket (KC); Performed the Experiments: MC (performed scent detection dog research) & KC (performed eDNA analysis); Analysed the data: MC, NL & TE; Interpretation of findings: MC; Wrote the paper: MC, CB, TE, GT, NL, DG.

2.1 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 are 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.

Keywords: Scent detection dogs; Environmental DNA; Invasive fish; Freshwater; Carp; Conservation

2.2 Introduction

Common carp (*Cyprinus carpio*) and the closely related koi carp (C*yprinus rubrofuscus*) are highly invasive fish species that have been introduced to freshwater ecosystems worldwide (McDowall, 1996; Hicks & Ling, 2015). Often referred to as "ecosystem engineers," carp are known to modify aquatic systems through their foraging behaviour (Matsuzaki *et al.*, 2009; Weber & Brown, 2009; Gozlan *et al.*, 2010). Carp forage in bottom sediments uprooting aquatic vegetation and re-suspending nutrient laden sediment into the water column (Bajer *et al.*, 2009; Weber & Brown, 2009; 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.*, 2009). Once carp populations have established, they can be difficult and costly to eradicate (Lougheed *et al.*, 2004; Weber & 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 & 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 from 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, dogs' 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.

2.3 Methods

2.3.1 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).

2.3.2 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 distractor 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 hours, 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.

2.3.3 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).

2.3.4 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 carp, 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 501 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 dogs 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 ≥80% hit rate for target samples and ≥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).

2.3.5 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 distractor), 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 2.1). Once in place, a stainless-steel lid was placed on the segments, and the samples were allowed to stand for 20 minutes to allow for release of VOCs before the session began. Each time the dog met an accuracy criterion of ≥80% hit rate on baseline and probe samples and a combined correct rejection rate (no fish + goldfish) of ≥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 2.3.4 (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.

Table 2.1: 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 concentration)	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

2.3.6 Environmental DNA

Comparative, laboratory-based eDNA 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 hour 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 8,000 rpm for 1 minute were completed to load a total of 1,200 µL of sample supernatant into the mini-spin columns. The samples were then washed by loading 500 µL of AW1 buffer, centrifuging at 8,000 rpm for 1 minute, then loading 500 µL of AW2 buffer, and centrifuging at 14,000 rpm for 3 minutes. The extracted DNA was then eluted in 200 µL of ultra-purified deionised water. Two negative extraction control samples were also included to monitor for potential contamination during the eDNA extraction process.

2.3.7 Assay Optimisation

A species-specific TaqMan assay designed and validated by Furlan and Gleeson (2016) to detect common carp (*C. carpio*12s) was used in this study. The assay targeted and amplified a 73 bp length fragment of mitochondrial DNA in the 12s gene region of the common carp mitochondrial genome (Table 2.2). 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.

Table 2.2**:** 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 12s gene region. From: Furlan & Gleeson (2016).

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

2.3.8 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).

2.3.9 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*12s-F, 1 µL of 10 µM of *C. carpio*12s-R, 1 µL of 10 µM of *C. carpio*12s-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 50ºC for 2 minutes, 95ºC for 10 minutes, followed by 50 cycles of 95ºC for 10 s, and 60ºC 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.

2.3.10 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 ≥80% and a combined correct rejection rate of ≥80% was achieved. All graphical and statistical analyses were performed using GraphPad Prism (Version 9).

2.4 Results

2.4.1 Scent Detection Dog Performance

In total, 106 sessions over 29 testing days (15 weeks) were conducted, taking an average of 7 minutes 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 (Figure 2.1A and 2.1B, 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.

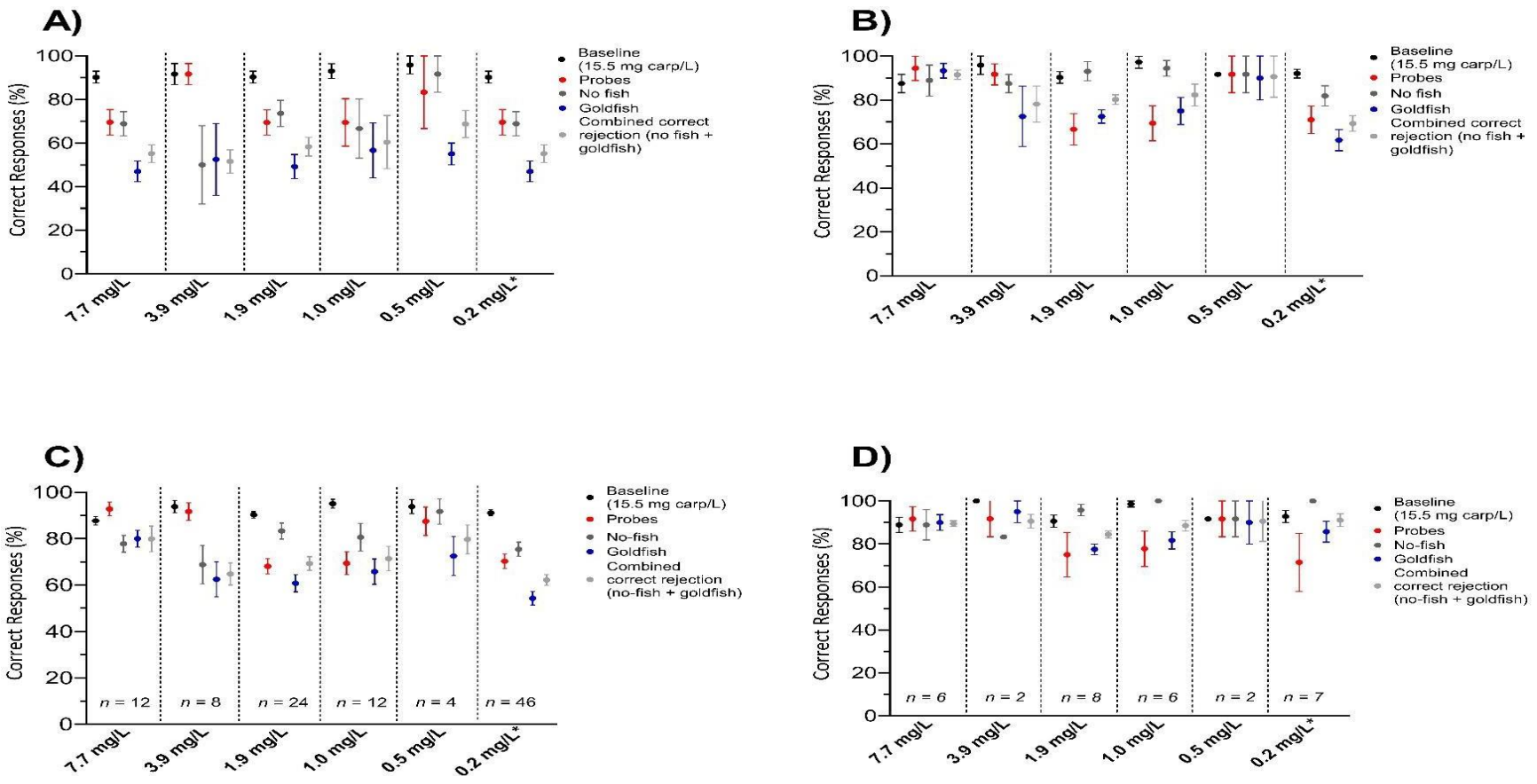

Figure 2.1: Hit rate on baseline and probe samples, correct rejection rate on goldfish and no fish samples, and combined correct rejection rate on both non-target samples (± standard error) in the first session performed by the dog on each sample array (A), second session (B), across all sessions (C) and sessions where she had a hit rate on baseline samples and a combined correct rejection rate of ≥80% was achieved (high accuracy sessions; D). 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. * Dog met termination criterion at this dilution.

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% to 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 (Figure 2.1C). 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) (Figure 2.1C).

Of the 106 sessions, only 31 (29%) were completed with a hit rate on baseline samples and combined correct rejection rate of ≥80% (hereafter, high accuracy sessions) (Figure 2.1D). When data from these high accuracy sessions were evaluated (Figure 2.1C) 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.

2.4.2 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, although PCR success rate significantly declined below 1,000 copies/µL (Figure 2.2). Limit of quantification, based on a 90% likelihood of a positive PCR reaction, was determined to be 1,300 copies/µL equating to a DNA concentration in water of 260,000 copies/L, assuming an extraction volume of 200 µL from the filter.

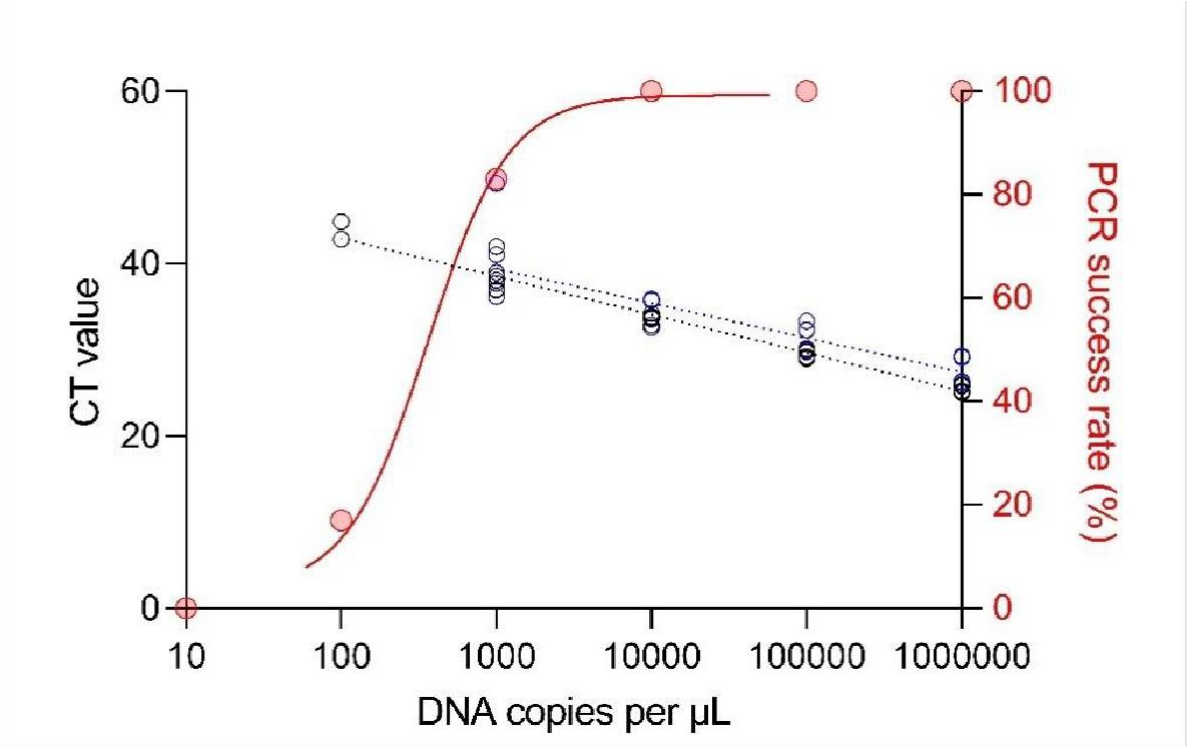

Figure 2.2: Calibration of qPCR against synthetic oligonucleotide concentration. Two qPCR calibrations were undertaken: blue and black open circles respectively. Number of DNA copies is the copy number per µL in sample added to the PCR: a total of 4 µL was included in the PCR reaction. PCR success rate is the cumulative success rate from both runs (red dots and line).

Comparison of normalised fish tissue biomass per litre with the qPCR CT values determined by the *C. carpio12*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 (Figure 2.3) 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*12s assay also amplified samples containing goldfish DNA but with far lower efficiency (Figure 2.3). No carp DNA was detected in any samples of control dechlorinated tap water up to filtered volumes of 10 L.

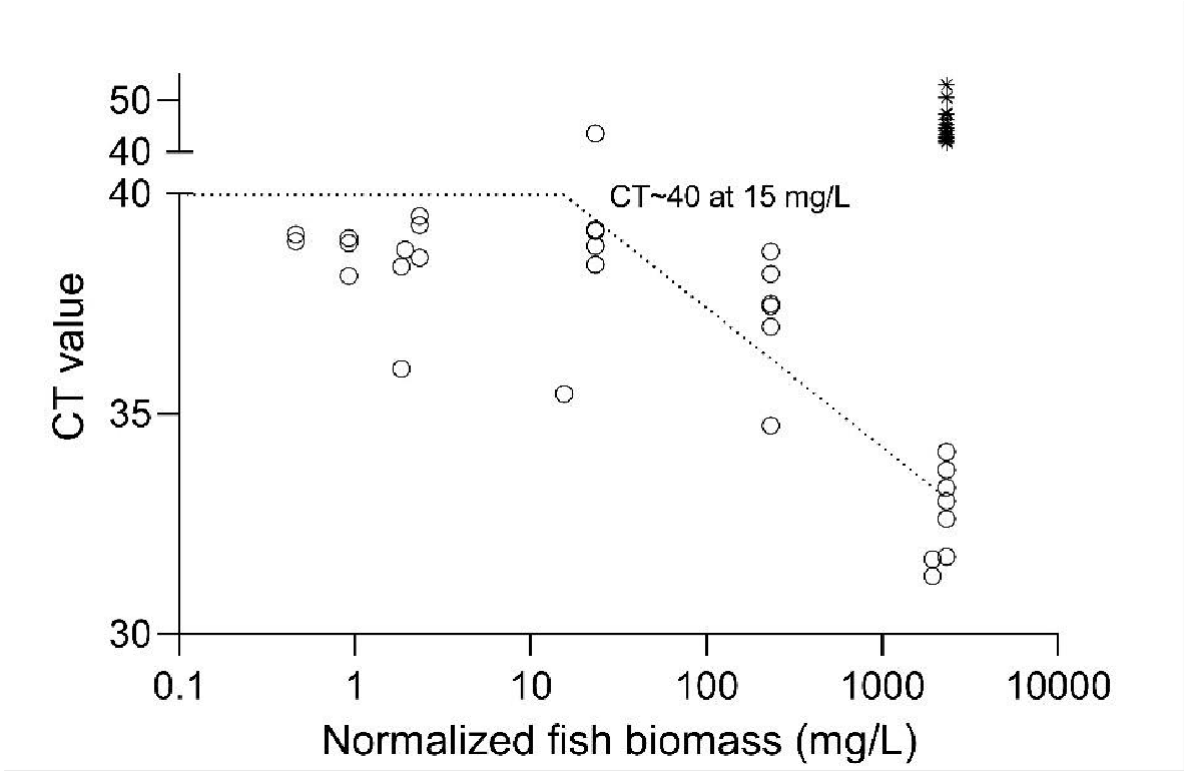

Figure 2.3 qPCR CT values compared with relative fish biomass using the *C. carpio*12s assay for carp (open circles) or goldfish (asterisks). CT values for samples below 10 mg/L are generally for samples with filtered water volumes greater than 1 L.

Comparison of calculated DNA copies per litre of filtered water with 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; Figure 2.3). 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 (Figure 2.4).

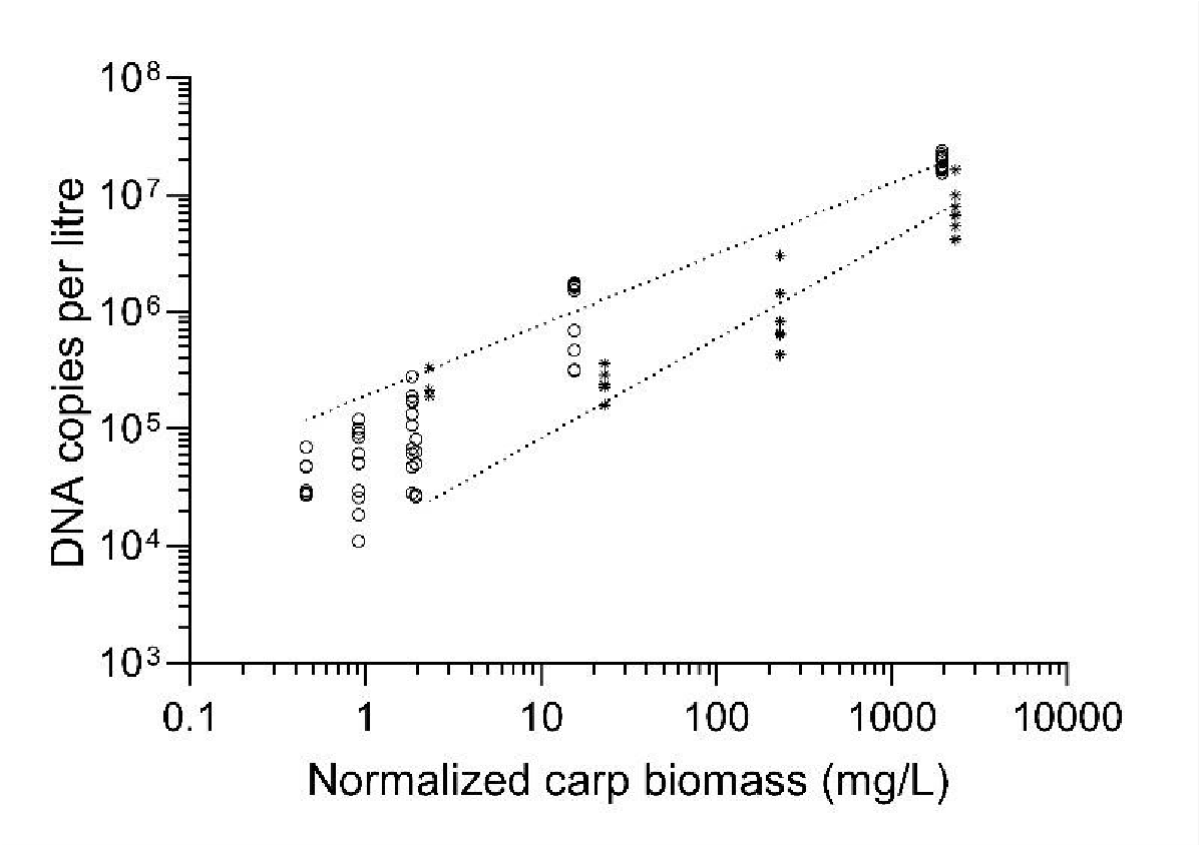

Figure 2.4: Comparison of normalized fish biomass and DNA copy number calculated from qPCR. Results include two separate fish biomass dilution series (open circles and asterisks, respectively). Labels indicate comparative detection thresholds for eDNA and dog scent detection.

2.5 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 & 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 were 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., 1.7 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 minutes). 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.

eDNA 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 & 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 & Grainger, 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; Jamieson *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 dog's ability to detect carp.

2.5.1 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.

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Chapter 3

Effects of preservation techniques on dogs' (*Canis familiaris*) ability to detect koi carp (*Cyprinus rubrofuscus*) scent from water samples

Scent detector dog Marley

Why is it important to apply preservation techniques to scent samples?

Chapter 2 revealed that the detection dog and eDNA had similar sensitivities to carp, with both methods detecting carp down to 9.3 kg/ha in aquaria water samples (Collins *et al.*, 2022[7]). While these results are promising, for canine scent detection to become a viable invasive fish detection method, dogs would have to assess samples that have been brought into the laboratory. To maintain the integrity of the samples from the time of collection to the time of analysis by the dogs, preservation methods would need to be applied to the water samples (Moldoveanu & David, 2002[8]; Schoon, 2005[9]; Forbes *et al.*, 2014[10]). Limited studies have investigated the impact different sample preservation methods have on dogs' detection performance (Chi *et al.*, 2021[11]; Matthew *et al.*, 2021[12]; Needs *et al.*, 2021[13]). In an attempt to determine the optimum preservation method for canine assessed water samples, Chapter 3 evaluated dogs' detection performance on water samples that had been held in a refrigerator (4°C), freezer (-18°C), or at room temperature with potassium sorbate added, for 7 or 8 days.

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Effects of preservation techniques on dogs' (*Canis familiaris)* ability to detect koi carp (*Cyprinus rubrofuscus*) scent in water samples

3.1 Abstract

Koi carp (*Cyprinus rubrofuscus*) are an invasive fish that has caused significant ecological impacts to New Zealand's freshwater ecosystems. Although several carp detection methods exist (i.e., observation, netting, electrofishing, and environmental DNA), they can be time-, labour-, and cost-intensive, especially when fish densities are low. Sending water samples to a laboratory for evaluation by scent detection dogs could offer a more efficient method for invasive fish detection. However, the odour profiles of such samples are likely unstable, and without effective preservation from the time of collection to the time of analysis by dogs, samples could degrade, making the target scent less recognisable to dogs. The aim of this study was to determine whether refrigeration (4°C), freezing (-18°C), or the addition of potassium sorbate (1.7 mM) could be used to preserve scent in water samples assessed by dogs. An ABACADA reversal design was employed. The dogs performed a baseline evaluation (A) of unpreserved water samples from aquaria containing carp (target scent; n = 7), goldfish (*Carassius auratus*; non-target distractor scent; n = 5), or no fish (non-target scent; n = 5) before preservation treatments were applied to the samples. The treatment phases (B = refrigeration, C = freezing, and D = room temperature with potassium sorbate) involved applying a preservation method to the water samples 7 or 8 days prior to assessment by the dogs. There was weak evidence that the dogs' detection performance was impaired on the refrigeration samples. In contrast, once stability criteria had been met, detection performances on frozen or potassium sorbate preserved samples were similar to or returned back to baseline levels. These findings suggest that freezing and potassium sorbate effectively retarded scent degradation over the storage period and thus have the potential to be used as preservatives for water samples that require assessment by scent detection dogs. If laboratory-based canine scent detection becomes an invasive fish detection method, the use of dogs could prove more time- and cost-efficient than current detection methods.

Key words: Conservation, Invasive fish, Carp, Water samples, Preservation, Dog, Scent Detection

3.2 Introduction

Dogs have an advanced olfactory system that is 10,000-100,000 times more sensitive than that of humans (Walker *et al.*, 2003; Walker *et al.*, 2006; Sankaran *et al.*, 2012). Due to their acute olfactory abilities and trainability, humans use dogs for a wide range of scent-detection tasks (Browne *et al.*, 2006). For example, dogs have been used to detect explosives, illicit substances, missing persons, cadavers, and various diseases (Browne *et al.*, 2006; Helton, 2009). Over the past century, dogs have also been used for conservation purposes, and have successfully detected numerous endangered or invasive species (Hill & Hill, 1987; Browne *et al.*, 2006; Beebe *et al.*, 2016).

Most research involving conservation detection dogs has been conducted in natural settings, where the dogs are taken to an area to detect *in situ*. However, in some cases samples have been collected from the environment and evaluated by trained dogs in the laboratory (DeShon *et al.*, 2016). Laboratory-based scent detection can have certain benefits over field surveys. For example, dogs can be trained to examine samples independently without a handler, thereby reducing the risk of unintentional cues (Edwards, 2019; Aviles-Rosa *et al.*, 2021). Researchers have greater control over environmental factors (e.g., temperature and humidity) that can influence dog olfactory performance (Reed *et al.*, 2011; Savidge *et al.*, 2011; Chambers *et al.*, 2015). Lastly, there is no need to transport dog handler duos to or from field sites, reducing the time spent travelling and improving efficiency. Despite these advantages, laboratory-based scent detection requires that the integrity of the sample is maintained from the time of collection to the time of analysis by the dogs.

Depending on the location of the field site and the number of sites assessed, it may take hours or days before samples can be returned to the laboratory and analysed by dogs. This delay can be problematic because physical (i.e., volatilisation) and biochemical processes (i.e., photodecomposition, microbial decomposition, oxidation, or reduction) within the sample can

alter the original scent profile, potentially resulting in a target scent that is dis-similar to the dogs training scent (Moldoveanu & David, 2002; Schoon, 2005; Forbes *et al.*, 2014; Chi *et al.*, 2021). An effective way to retard scent degradation is to use preservation methods.

Refrigeration and freezing are the primary methods used by scent detection researchers to preserve scent samples (e.g., Chi *et al.*, 2021; Matthew *et al.*, 2021; Crawford *et al.*, 2022). These methods retard the rate at which chemical and biological reactions occur and have proven effective at maintaining the olfactory profile of a sample for extended periods (>7 days) of time (Chi *et al.*, 2021). Despite their efficacy, it can be difficult to transport or use cold storage devices in remote areas with limited vehicle access. Hewitt (1995) investigated chemical preservation as a way of inhibiting biological degradation of volatile organic compounds, specifically toluene and benzene, in soil samples. The results revealed that in the presence of methanol and sodium bisulphate the soil samples could be held at room temperature for up 14 days without any significant changes in volatile composition. In contrast, when samples were immersed in water and held at 4°C, the concentration of toluene and benzene began to decrease after day two. Despite these promising results, very few studies have explored chemical compounds and their potential as a preservative for canine assessed samples.

Koi carp (*Cyprinus rubrofuscus*) is an invasive fish that has contributed to the decline of many of New Zealand's freshwater ecosystems (Rowe, 2007; Schallenberg & Sorrell, 2009; Collier & Grainer, 2015). Although there are various carp detection methods (i.e., observation, netting, electrofishing, and environmental DNA), they can be time-, labour-, and cost-intensive, especially when fish densities are low (Evans *et al.*, 2017; Hinlo *et al.*, 2017; Furlan *et al.*, 2019). Sending water samples to be evaluated by trained scent detection dogs in a laboratory could offer a more efficient method of invasive fish detection. However, preservation would be necessary to keep the original olfactory profile of the samples intact between the time of collection to the time of evaluation by dogs. The aim of this study was to assess the effects of

refrigeration (4°C), freezing (-18°C), or the addition of potassium sorbate (1.7 mM), a chemical compound commonly used to preserve human food, on dogs' ability to detect carp scent (Baker & Grant, 2018). The most effective preservation technique could then be applied to water samples that require assessment by dogs.

3.3 Methods

3.3.1 Subjects

Of the 15 dogs initially trialled for this study, four female dogs between 3 and 9 years of age completed the training stage and participated in this experiment (Table 3.1). These dogs were brought into the scent detection research facility at the University of Waikato, New Zealand, twice a week for experimental testing. When not participating in the experiment, the dogs were individually housed in crates containing bedding and a water bowl. Dogs were not housed overnight, and all experimental procedures were approved by the University of Waikato Animal Ethics Committee (protocol #1013).

Table 3.1: Details of the subjects involved in the study.

Dog	Sex	Breed	De-sexed	Age (years)
Sabi	Female	Labrador retriever	Yes	8
Ruby	Female	Labrador retriever X border collie	Yes	9
Harlee	Female	Labrador retriever	Yes	3
Marley	Female	Siberian husky cross	Yes	7

3.3.2 Fish Husbandry and Sample Collection

Water samples were collected from three high-density polyethylene tanks (195 L) fitted with lids, housing either carp (target scent; $n = 1$), goldfish (*Carassius auratus*; non-target distractor scent; $n = 4–5$ fish depending on fish size), or no fish (non-target scent). Goldfish was selected as the distractor fish 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. During the non-experimental periods,

the tanks were supplied with continuous flow-through dechlorinated tap water, and the oxygen levels were maintained via compressed air. Twenty-four hours prior to sample collection, the water flow into each tank was halted, the tanks were partially drained, and the interior was scrubbed clean and flushed with water. The carp and goldfish tanks were then refilled to achieve a standard fish biomass of 15.5 g/L with no flow-through. The 'no fish' tank remained on flow-through.

After 24 hours, tank water samples were collected using individual 68% nitric acid-cleaned beakers and transferred into species-specific 10% v/v hydrochloric acid-washed 1 L Nalgene bottles. The water samples were then immediately transported to the scent detection facility and used for sample preparation.

To help prevent recognition of unique odour signatures and to remove excreta and organic matter from the tanks, a deep clean of the experimental tanks was performed at the start of each month. During this procedure, the fish were removed from tanks, and the experimental tanks were drained, scrubbed, and sprayed with 10% v/v hydrogen peroxide. After 20 minutes the tanks were returned to flow-through for 24 hours before a new set of fish were placed the tanks. The fish were changed a total of nine times throughout the duration of this 9-month experiment.

3.3.3 Scent-detection Apparatus

An automated rotating carousel containing 17 removable aluminium segments was used to present the samples to the dogs (Figure 3.1). The dog accessed each segment through a 10 cm circular opening in a panel at the front of the apparatus. There was a flap at the front of each segment that moved inwards when a dog assessed a sample, and then closed once the dog removed its nose (Figure 3.1). When the dog assessed a sample, infrared beams behind the port were broken, initiating an auditory 'beep' and the automatic recording of the dog's assessment duration. If the dog held its nose in the port for a prespecified time (4 s) and the segment

contained the target scent (carp scent), a nearby wireless feeder delivered kibble as reinforcement. This was recorded by the researcher as a hit (i.e., the dog correctly indicated that the target signal [carp] is present). On non-target samples (goldfish and no fish), the dog was required to assess the sample for a minimum period of 0.5 s and then press a switch on the side of the apparatus; this switch rotated the carousel and presented the dog with another water sample. If the dog assessed a non-target sample for more than 0.5 s but less than 4 s, a correct rejection was recorded (i.e., the dog correctly identified that the signal, goldfish or no fish, was not the target scent). False alarms were recorded when the dog assessed a non-target sample for more than 4 s (i.e., incorrectly identified a non-target scent as the target signal), whilst misses were recorded when the dog assessed a target sample for less than 4 s and then pressed the switch (i.e., the dog failed to indicate the target signal).

The apparatus, feeder, and cameras used to observe the trials were run by computers equipped with custom-made software outside of the experimental room. A full description of the apparatus design and operation can be found in Edwards (2019). This approach allowed the dog to work without a handler being present in the room, reducing the potential influence of human cueing and subjectivity.

Figure 3.1: Automated scent-detection apparatus showing (A) the opaque plexiglass front panel, porthole and omnidirectional switch, (B) the segment flap, and (C) the 17 segments containing the various target and non-target samples.

3.3.4 Dog Training

The method of reinforcing successive approximations of the final behaviour (i.e., shaping) was used to train the dogs to operate the scent detection apparatus independently of their handler (Skinner, 1975; refer to Appendix C, section 2 for the shaping procedure). Once autonomous operation of the scent detection apparatus was achieved, the dog was exposed to a dilution series (Table 3.2). Each time the dogs meet an accuracy criterion of 4/5 consecutive sessions (session = assessed all 17 samples, once) with a hit rate of ≥80% and a correct rejection rate on goldfish and no fish samples (individually) of ≥80%, the samples were systematically diluted by 50%. A more detailed explanation of the training procedure is outlined in Chapter 2. The dogs entered the preservation experiment once they had achieved the accuracy criterion at a dilution equivalent to 621 kg carp/ha, assuming a 1 ha by 2 m deep water body. Although this is a comparatively high biomass of carp, such densities have been reported in natural aquatic systems (Fletcher *et al.*, 1985; Driver *et al.*, 1997; Haque *et al.*, 2007; Vilizzi *et al.*, 2015). We chose a high biomass of carp to ensure that the dog's performance was based on the impact of preservation, rather than their inability to detect carp due to a low biomass.

Table 3.2: Training dilutions and their equivalent biomass relative to volume (mg/L) or area (kg/ha).

Trained Dilution	Biomass (mg/L)	Biomass (kg/ha*)
1	5,188	103,764
2	3,112	62,258
3	1,729	34,588
4	915	18,311
5	453	9,066
6	230	4,600
7	123	2,470
8	62	1,240
9	31	621

*kg/ha measure based on a water volume of 20,000 m^3 per hectare i.e., 100 m x 100 m x 2 m water depth.

3.3.5 Experimental Design

An ABACADA reversal design was used for this study. The dogs performed a baseline evaluation (A) of unpreserved water samples from aquaria containing carp (target scent; $n = 7$), goldfish (non-target distractor scent; $n = 5$), or no fish (non-target scent; $n = 5$) before preservation treatments were applied to the samples. The treatment phases (B, C, and D) involved applying a preservation method to the water samples 7 or 8 days prior to assessment by the dogs.

3.3.6 Experimental Procedure

The baseline unpreserved samples were prepared by filling the 17 plastic presentation containers with 100 mL of fresh (<1 hour old), no-fish water from the control aquarium. Water sourced from either the goldfish or carp aquaria was added to five and seven of the containers, respectively, to achieve a standard fish biomass of 31 mg/L (equivalent to 621 kg/ha) while the remaining five were left fish-free. For preparation of the preserved samples, six Nalgene bottles were filled with 1 L of aquaria water containing no fish scent. Four bottles were then spiked with either carp ($n = 2$ bottles) or goldfish ($n = 2$ bottles) aquaria water to achieve a fish biomass of 31 mg/L, while the remaining two Nalgene bottles remained free of fish scent. The prepared samples were then placed in either a refrigerator (4°C), freezer (-18°C), or dark cupboard at room temperature with potassium sorbate (1.7 mM) for 7 or 8 days. To ensure that all samples were presented to the dogs in liquid form, the frozen samples were defrosted at 21°C 24 h prior to experimental testing. Once the storage period had elapsed, plastic containers were filled with 100 mL of preserved carp ($n = 7$), goldfish ($n = 5$) or no fish ($n = 5$) water and placed in their segments on the scent detection apparatus. A lid was placed on top of the segments and the samples were left to sit for 20 minutes to allow VOCs released from the samples to equilibrate with the segments' headspace.

Following the 20-minute sit time, the dogs entered the experimental room individually to perform a session, where they assessed the 17 samples consecutively. The dogs performed 2-6 sessions with the apparatus presenting the samples in a clockwise direction, and 2-6 sessions with the apparatus presenting the samples in an anticlockwise direction. The bi-directional presentation of samples, as well as 5-minute breaks between sessions, were employed to help prevent the dogs from learning the location of the targets on the carousel, as rearrangement of samples after each session was not feasible. A maximum of 12 sessions were performed on an experimental day. If the researcher thought a dog lacked motivation to work or was fatigued, the number of sessions performed in an experimental day was reduced. Hits, misses, correct rejections, and false alarms were recorded and used to measure the dogs' detection accuracy. The dogs only received reinforcement (i.e., kibble) from the automated feeder when a hit was performed.

To aid the progression of the experiment, all dogs graduated to the next phase of the experiment each time Sabi (the only dog to participate in all stages of the experiment) reached a stable state of performance. The performance of a dog was considered stable when their daily average hit rate on target samples and their correct rejection rate on non-target samples (goldfish and no fish, individually) from the two most recent experimental days did not differ by more than 10% from those measured from the two prior experimental days (Perone & Hursh, 2013).

3.3.7 Statistical Analysis

Daily sensitivity and specificity values were calculated for each dog using the method outlined by Trevethan (2017). These values were then graphed to evaluate the dogs' detection performance across the different phases before the stability criteria were met and while they

were being met. The dogs' mean detection performance on each phase where they had reached a stable state of performance was also graphed.

Tau-U is a quantitative approach for analysing single-case experimental designs. It works by combining non-overlap between phases with the intervention phase trend and can correct for baseline trend (Lee and Cherney, 2018). To determine the effects of each treatment on the dogs' detection performance, Tau-U analysis was performed on the stable state data using a web-based application (Vannest *et al.,* 2016). If a dog had met criteria on baseline samples before and after a treatment, data from both baselines were combined so that AB comparisons could be made. The slope of the line was then calculated. No baseline slopes demonstrated a significant difference ($P < 0.05$), therefore no corrections for a baseline trend were performed (Brossart *et al.*, 2018). When interpreting the results, we followed the Vannest and Ninci (2015) suggestion for labelling Tau in the range of 0–0.20 as small change, 0.20–0.60 a moderate change, 0.60–0.80 a large change, and >0.80 a very large change in performance.

To determine if the dogs' performance was above chance levels, expected chance performance was calculated by dividing the total number of target or non-target samples by the total number of samples available for assessment. For example, if seven of the 17 available water samples contained the target scent, then the dog would have a 41% chance of indicating a target correctly. Ninety-five percent confidence intervals (based on a binomial distribution) were then applied to each dog's mean performance, aggregated across all conditions for each sample type. A dog was considered to have performed above chance levels if the value at the lower 95% confidence interval limit was greater than the calculated value of expected chance performance. All graphs and descriptive statistics were made using GraphPad Prism (Version 9).

3.4 Results

Two dogs participated in the first two phases (Baseline 1 and Refrigeration), while four dogs participated in the remaining phases (Baseline 2, Potassium sorbate, Baseline 3, Freezing, Baseline 4) (Table 3.3). The difference in subject number across phases was a result of Harlee and Marley completing their training mid-way through experimental testing.

Table 3.3: Stability criterion met (✓) or not met (-) at each Baseline or treatment phase (Refrigeration, Potassium sorbate, Frozen). Blanks indicate that the dogs did not participate in the phase.

Dog	Sample Type						
	Baseline1	Refrigeration	Baseline 2	Potassium sorbate	Baseline 3	Frozen	Baseline 4
Sabi	✓	✓	✓	✓	✓	✓	✓
Ruby	-	✓	✓	✓		-	-
Harlee			✓	-	✓	✓	-
Marley			-	-	✓	✓	-

The dogs' daily average sensitivity and specificity across the different baseline and preservation phases both before stability criteria was met, and when it was met, is presented in Figure 3.2. The dogs' hit rates remained high across all baseline and preservation phases; however, false alarm rates were more variable. Notably, Sabi's false alarm rate on goldfish samples increased during the refrigeration phase and remained elevated and highly variable in the subsequent baseline phase (i.e., Baseline 2). This trend was not observed with Ruby who participated in the same phases, as her false alarm rate was initially high and variable in the first baseline phase but improved during the refrigeration phase. When Ruby was returned to baseline samples her false alarms on goldfish samples decreased, while there was no notable change in Ruby's false alarm rate on the no fish samples (Figure 3.2).

The false-alarm rates of most dogs increased following the phase change from Baseline 3 samples to potassium sorbate-treated samples. However, as the dogs' exposure to potassium sorbate-treated samples increased, their false alarm rates tended to decrease. Variable results

were observed when the dogs were returned to Baseline 3; Sabi's false alarm rate on goldfish samples continued to trend downwards, Harlee's false alarm rates remained similar to baseline, while Marley's false alarm rates increased. Illness prevented Ruby from participating in Baseline 3 (Figure 3.2).

Comparisons between dogs' performance on Baseline 3 samples and frozen samples suggest that freezing samples had no major impact on the dogs' ability to discriminate carp from goldfish or no fish scent; dog detection performance remained similar or was better on frozen samples. When the dogs returned to baseline (Baseline 4), their false alarm rates tended to remain similar to or lower than their false alarm rates on frozen samples (Figure 3.2).

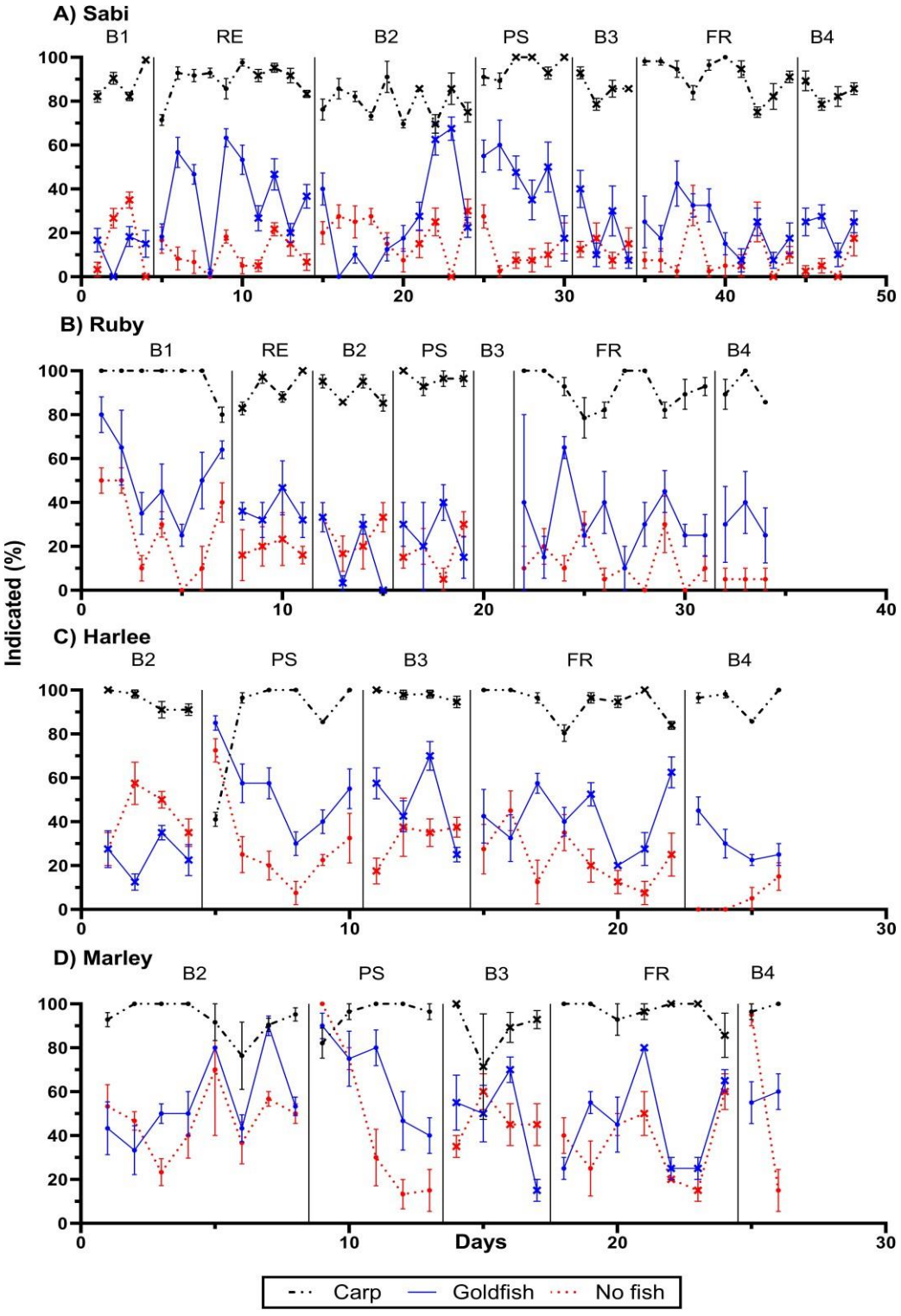

Figure 3.2: Individual dogs' daily average (mean ± standard error) hit rate on carp water samples (black), and false alarm rate on goldfish (blue) or no fish (red) water samples in each baseline (B1 = Baseline 1; B2 = Baseline 2; B3 = Baseline 3; B4 = Baseline 4) and preservation treatment phase (RE = Refrigeration; PS = Potassium sorbate; FR = Freezing). Lines with 'x' as the symbol indicate data where dogs met stability criterion. The blank sections in Ruby's graph are when she did not participate in the phase due to illness.

The performance means presented in Figure 3.3 were calculated exclusively from data where stability criteria had been met. Sabi's mean false alarm rate on goldfish samples increased from 12.5% to 32.5%, when she was introduced to refrigerated samples. This elevated false alarm rate persisted during the subsequent Baseline 2 phase, with Sabi experiencing an additional 12% increase in mean false alarm rates on goldfish samples. Despite not meeting stability criteria in the initial Baseline 1 phase, when Ruby was withdrawn from working with refrigerated water and returned to Baseline 2 samples her false alarm rate on goldfish decreased from 37.1% to 16.7%. The presentation of potassium sorbate samples caused Sabi's hit rate to increase from 79% to 98.2%, but only caused her false alarm rate on goldfish samples to decrease from 45% to 37.5% and her false alarm rate on no fish samples to decrease from 17.5% to 10.6%. Finally, freezing did not appear to have a major impact on dogs' detection performance, with all dogs except Harlee showing a ≤10% change in hit or false alarm rates, relative to their performance on Baseline 3 samples (Figure 3.3).

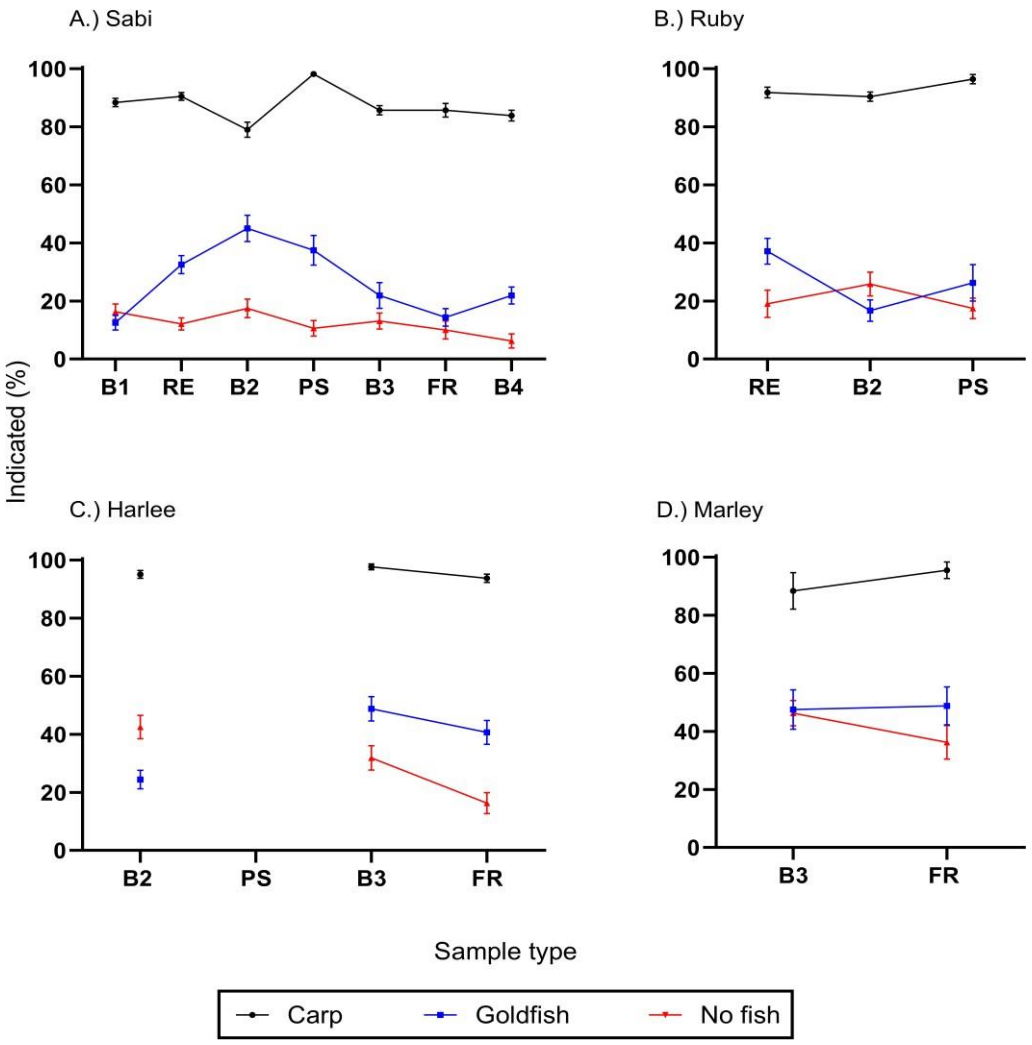

Figure 3.3: Mean (± standard error) hit rate on carp and false alarm rate on goldfish and no fish scent for individual dogs (A-D) on each baseline (B1 = Baseline 1; B2 = Baseline 2; B3 = Baseline 3; B4 = Baseline 4) and treatment (RE = Refrigeration; PS = Potassium Sorbate; FR = Frozen) phase from data where stability criterion was met.

Tau-U scores and associated *p*-values for each dog are presented in Table 3.4. When Sabi's pre- and post-baseline data was combined and compared to her performance on samples preserved with potassium sorbate, a significant difference in hit rate (Tau-U = 0.97; $p = 0.01$) was revealed. Although Sabi's false alarm rate on goldfish increased substantially in the presence of refrigerated samples, it increased further in the subsequent baseline phase. Thus, when pre-and post-baseline data were combined, no significant difference in detection performance was found. No other dogs demonstrated significant changes when AB or BA comparisons were performed.

Table 3.4: Tau-U scores and associated *p*-values for each sample type (carp, goldfish, no fish) where the stability criterion had been met. Following the recommendation of Vannest & Ninci (2015) the degree of performance change was considered small if the Tau-U score ranged between 0 and 0.20, moderate if it ranged between 0.20 to 0.60, large if it ranged between 0.60 and 0.80, and very large if it was >0.80. Significant changes in performance are signified with an asterisk.

Dog	Treatment	Carp			Goldfish			No fish		
		Tau	Degree of performance change	*p*-value	Tau	Degree of performance change	*p*-value	Tau	Degree of performance change	*p*-value
Sabi	Refrigeration [a]	0.56	Moderate	0.13	0.31	Moderate	0.40	-0.16	Small	0.67
	Potassium sorbate [a]	0.97	Very large	0.01*	0.19	Small	0.61	-0.34	Moderate	0.35
	Frozen [a]	0.09	Small	0.80	-0.50	Moderate	0.17	-0.06	Small	0.87
Ruby	Potassium sorbate	0.75	Large	0.08	0.31	Moderate	0.47	-0.56	Moderate	0.19
	Refrigeration [b]	-0.25	Moderate	0.56	-0.75	Large	0.08	0.56	Moderate	0.19
Harlee	Frozen	-0.38	Moderate	0.39	-0.25	Moderate	0.56	-0.75	Large	0.08
Marley	Frozen	0.38	Moderate	0.39	0.13	Small	0.77	-0.19	Small	0.67

[a] Baseline data from before and after the treatment were combined to allow for AB comparisons to be made.

[b] Intervention-Baseline (i.e., BA) comparison.

Sabi and Ruby performed above chance levels on carp, no fish and goldfish samples (Figure 3.4). Harlee performed above chance levels on carp and no fish samples, but not on goldfish samples. Marley's performance was below chance performance on both no fish and goldfish water samples (Figure 3.4).

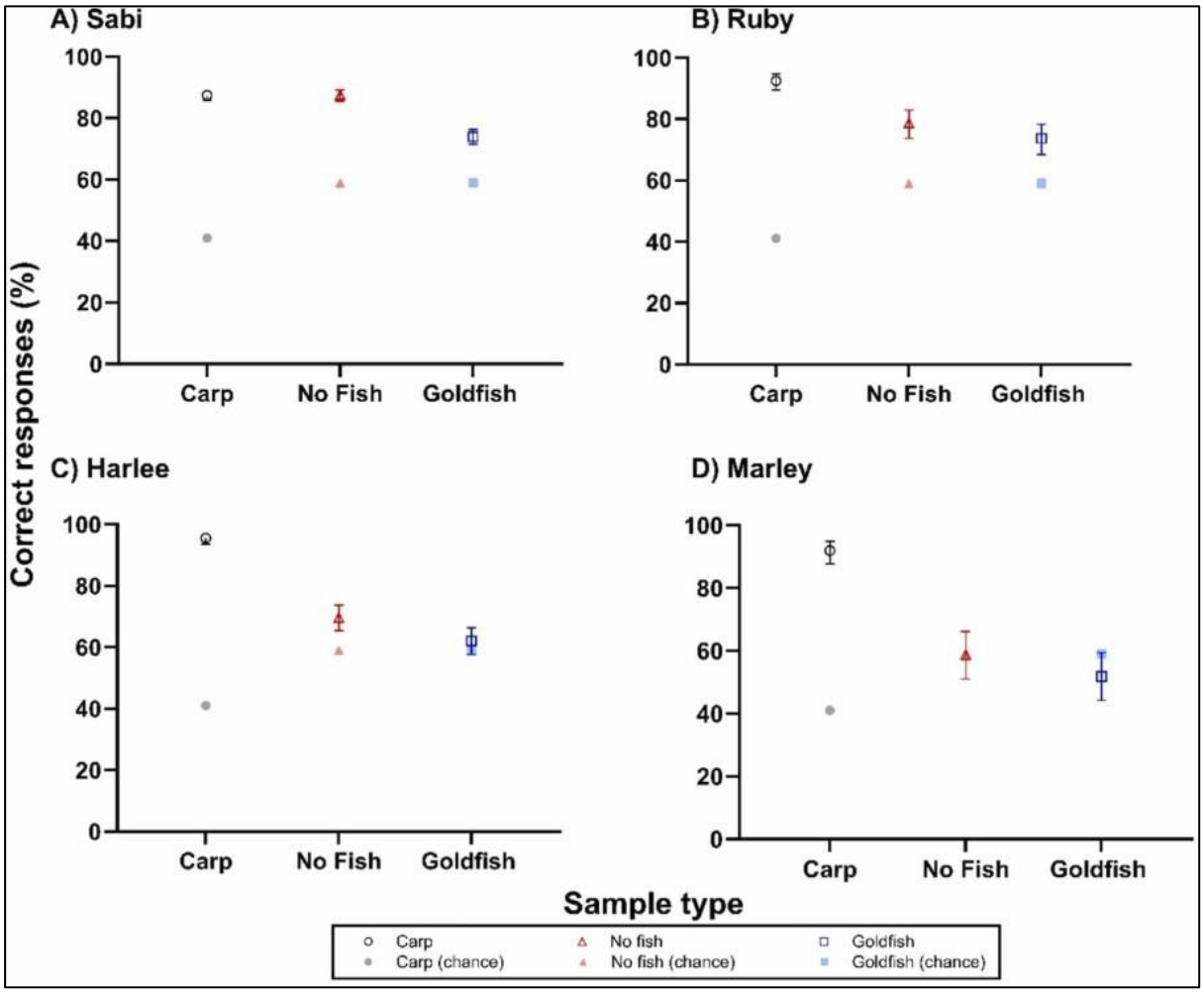

Figure 3.4: Performance that would be expected at chance level, and the dogs' mean percentage of correct responses (± 95% confidence intervals) on carp, goldfish and no fish samples once they had reached a stable state of performance.

3.5 Discussion

If canine scent detection were to become an established invasive fish detection method, water samples could be collected in the field and transported back to the laboratory. To prevent scent degradation during transit to the laboratory an effective preservation method would need to be applied. The aim of this study was to determine whether refrigeration (4°C), freezing (-18°C), or the addition of potassium sorbate (1.7 mM) could be used as preservation methods for water samples assessed by scent detection dogs.

Analysis of the dogs' stable state data revealed no significant differences in hit or false alarm rates when the dogs were introduced and/or withdrawn from refrigerated samples. Notably, Sabi's false alarm rate on goldfish samples increased during the refrigeration phase, but continued to increase in the subsequent baseline phase, suggesting that factors other than the treatment may have been responsible for the observed change in performance. Ruby did not meet stability criteria in the first baseline phase, so definitive conclusions about the effects of refrigeration on Ruby's detection performance cannot be drawn. However, there was a large decrease in her false alarm rate when she was withdrawn from refrigerated samples and returned to baseline. While these results provide limited evidence regarding the impact of refrigeration on dogs' detection performance, early indications are that refrigeration may not be an effective preservation method for water samples assessed by dogs.

When the dogs were exposed to potassium sorbate-preserved samples, they initially showed an increase in hit and false alarm rates. However, as the dogs continued to only receive reinforcement for indicating on carp samples, their false alarm rates gradually decreased. This trend suggests that the dogs may have undergone error-driven learning (Soto & Wasserman, 2010). In other words, through constant reinforcement on carp samples only, the dogs were able to learn that carp samples remained the target, even when potassium sorbate was present. Statistical analysis of the dogs' stable state data revealed that potassium sorbate had no

significant adverse impacts on the dogs' detection performance. In fact, it was accompanied by a significant increase in one dog's hit rate (i.e., Sabi) and a large, but not statistically significant, increase in another (i.e., Ruby). This suggests that potassium sorbate has the potential to be used as a preservative for canine-assessed water samples. Potassium sorbate could improve researchers' ability to collect water samples in remote locations as this preservative is readily available and permits storage of samples at room temperature.

Other studies have demonstrated that freezing samples can effectively maintain the original olfactory profile of a scent for extended periods. For example, Needs *et al.* (2021) revealed that dogs can detect frozen and live plant material with comparable accuracy, achieving sensitivity levels of >85% and precision levels of 100% for both sample types. Moreover, Chi *et al.* (2021) demonstrated that dogs' positive indication rates on frozen fire ant (*Solenopsis invicta*) samples remained >90% and did not differ significantly from their positive indication rates on fresh, unpreserved samples <1 day old. Our results also indicate that freezing is an effective preservation method, as no dog's detection performance changed significantly from baseline when they were presented with samples that had been frozen for 7 or 8 days.

Assessment of the dogs' performance on target and non-target samples irrespective of treatment type revealed that Ruby and Sabi performed above chance levels on carp, no fish, and goldfish samples. In contrast, Harlee's performance was no better than chance on one non-target sample type (goldfish), while Marley's performance was no better than chance performance on both non-target sample types (no fish and goldfish). These differences could be related to: (1) individual differences in the dogs' scenting abilities (Polgár *et al*., 2016) or (2) experience with the target scent. Sabi and Ruby had a longer history of training and met criteria much earlier than Harlee and Marley. This increased exposure to the target scent could have improved their ability to discriminate carp from non-target scents.

Despite the insights on preservation efficacy, the sample size was comparatively small and variable across the different experimental phases. This variability was a by-product of Harlee and Marley's non-participation in the first two phases, as they only completed their training midway through the experiment. Moreover, while the decision to progress all dogs to the next phase once Sabi had reached a stable state of performance aided progression of the study, it meant that some dogs did not meet stability criterion in certain phases.

Differences in indication times on target and non-target samples, and the inability to clean or replace the segments or samples after each session or each dog may have influenced the data. Longer sniff durations for target samples could have led to more dog-sourced VOCs being left in the odour chamber, potentially contaminating the scent. Consequently, the dogs could have used their own cues or other dogs' cues to enhance their detection performance in subsequent sessions (Ferry *et al.*, 2019). Randomising the order that the dogs perform their sessions and monitoring their detection performance to determine if order effects are occurring should be considered for future studies. Alternatively, replacement of all segments and samples after each session would be the optimal solution.

When performing a search, dogs are often taught to sit, lie down, or scratch when they 'detect' the target scent. In this study, sniff duration was used as the indication behaviour. While the apparatus accurately measured the dogs' sniff duration, dogs have been known to assess novel stimuli for longer durations than familiar stimuli (Gadbois and Reeve, 2014). Consequently, it is possible that the dogs evaluated the water samples for longer on their first encounter, potentially leading to higher false alarm rates in their first session.

It has been demonstrated that dogs can detect carp down to a biomass equivalent to 9.3 kg/ha in carbon-filtered aquaria water (Collins *et al.*, 2022). However, dogs' detection threshold on preserved samples needs to be evaluated. This is particularly important for chemical preservatives like potassium sorbate which could release a scent that masks or overpowers

lower concentrations of carp scent, potentially impacting dogs' potential as an early detection method.

Limited studies have investigated how long preservation methods can maintain the integrity of the sample. Chi *et al.* (2021) demonstrated that dogs' positive indication rates on samples refrigerated or frozen for 13 weeks did not differ significantly from their positive indication rates on unpreserved samples <1 day old. In contrast, Matthew *et al.* (2021) reported that independent of the preservation method, the dogs' sensitivity to bullfrog scent (*Pyxicephalus adspersus*) was significantly higher after 1 month of preservation than 6 months of preservation. Given that preservation methods slow, but do not prevent scent degradation, the length of time water samples may be stored requires further investigation.

Future studies should also perform gas chromatography-mass spectrometry on un-preserved, frozen, refrigerated, and potassium sorbate treated water samples held for different storage durations. This would allow the researcher to evaluate how the VOC profile of unpreserved samples changes with time, and how effective each preservation treatment is at preventing these changes from occurring. It would also allow the researcher to gain an idea of what volatile components the dogs may be using to detect carp presence.

This experiment was a step towards the larger goal of using laboratory-based dogs as a tool to detect new incursions of invasive carp. Given that water samples collected from natural ecosystems (e.g., lakes, rivers, or ponds) may contain more non-target odours (e.g., VOCs released from aquatic plants, other fish species, amphibians, reptiles, birds, mammals, and chemical pollutants) than water sourced from laboratory aquaria, it is essential that dogs' detection performance on naturally sourced water is evaluated before they are deployed as a carp detection method. Additionally, the dogs' responses to intermittent reinforcement on carp water samples would also need to be assessed before their deployment. If water samples were

to be sent to the laboratory for evaluation by dogs, the status of those samples would be unknown, thus reinforcement for correct indications on those samples would not be possible.

3.5.1 Conclusion

This research corroborates the findings of other scent detection studies, which have revealed that freezing may preserve the integrity of the scent sample for extended periods (Chi *et al.*, 2021, Needs *et al*., 2021, Matthew *et al*., 2021). In addition, this research has demonstrated for the first time that potassium sorbate may be a viable preservation technique for water samples assessed by trained carp detection dogs. While further research is required, the use of laboratory-based scent detection dogs could not only represent another non-invasive approach to invasive fish detection, but it could also improve researchers' ability to rapidly and extensively survey waterbodies.

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Chapter 4

Canine scent detection of invasive fish, *Cyprinus rubrofuscus*, from lake water samples

Trainee scent detector dog Aspen

Why evaluate dogs' sensitivity and specificity to carp in naturally sourced water?

The results from the previous two chapters demonstrated that scent detection dogs can detect carp from fresh and preserved water samples collected from laboratory aquaria. Moreover, those studies also showed that dogs can discriminate carp from two non-target scents: goldfish scent and no fish scent. However, if dogs were to be used as an early detection method, they would be required to detect carp from water sourced from natural aquatic systems, which would contain more background odourants than carbon-filtered aquaria water. Depending on the saliency of these odours they could impact dogs' sensitivity and specificity to carp, potentially effecting dogs' overall viability as an early detection method. To determine if dogs have potential to provide an accurate and highly sensitive surveillance method for carp in real world circumstances, Chapter 4 assessed dogs' ability to detect carp in lake water. Environmental DNA analysis was also performed on the same samples assessed by the dogs to compare the detection performance of both methods.

Canine Scent Detection of Invasive Fish, *Cyprinus rubrofuscus*, from Lake Water Samples

4.1 Abstract

Carp (*Cyprinus* spp.) are an invasive freshwater fish species that have facilitated significant declines in water quality following their introduction. Early detection of carp invasions can improve chances of control and eradication, but current survey methods are often resource-intensive and have reduced sensitivity at low population abundance. This study evaluated dogs' (n = 3) potential as a surveillance method for carp by performing two experiments which assessed their ability to detect carp in water sourced from natural lakes. Experiment One's results indicated that dogs could accurately detect a standard biomass of carp (~310 kg carp/ha) in lakes of various trophic states, with the dogs yielding sensitivity values of >83.6% and specificity values of >77.3% on all lakes tested. When task complexity increased and the dogs assessed a sample array that contained lake samples that differed in water quality, familiarity, and carp biomass their detection accuracy decreased and, in some instances, failed to exceed chance performance. In comparison, eDNA analysis of the same water samples assessed by the dogs revealed that the multispecies assay detected carp in three out of the five lakes known to contain carp scent. Quantitative PCR using a species-specific primer improved detection of carp, yielding positive detections for four out of the five lakes, but still failed to detect carp in a Lake Waahi, which is known to contain a naturalised carp population. In summary, further research is warranted to determine the extent of dogs' ability to generalise carp scent due to COVID-19 restrictions limiting data collection. If dogs prove successful at detecting different densities of carp across a range of water qualities and amongst a variety of background odourants, they could provide a rapid and cost-efficient method of invasive fish detection. This could improve researchers' ability to survey lakes and other aquatic systems.

Keywords: aquatic ecosystems, conservation, carp, freshwater, invasive fish management, environmental DNA, genetics, scent detection dogs

4.2 Introduction

Freshwater ecosystems are prone to biodiversity loss, with the greatest threats coming from land use change, habitat loss, climate change, overfishing, and invasive species (Havel *et al.*, 2015; Arthington *et al.*, 2016). With increasing trade and international travel, numerous species have been introduced to areas outside of their natural range (Francis & Chadwick, 2012; Arthington *et al.*, 2016). Although most species fail to establish, those that succeed often have significant ecological or economic impacts (Francis & Chadwick, 2012).

Koi carp (*Cyprinus rubrofuscus*) were introduced to New Zealand in the early 1960s and are now found in freshwater ecosystems throughout the North Island (McDowall, 1996). Carp are considered a noxious, unwanted species in New Zealand due to their destructive feeding behaviour, which uproots macrophytes and resuspends nutrient-laden water in the water column (Collier & Grainger, 2015). When carp biomass reaches a critical threshold (e.g., >100 kg/ha), they can shift entire ecosystems from clear macrophyte-dominant state to a turbid phytoplankton-dominant state (King *et al.*, 1997; Chumchal *et al.*, 2005; Driver, 2005; Vilizzi *et al.*, 2014; Bajer & Sorensen, 2015; Collier & Grainger, 2015).

Early detection and the prompt implementation of control measures are essential in preventing invasive species, like carp, from establishing populations and causing significant ecological impacts (Lodge *et al.*, 2006; Collier & Grainger, 2015; Larson *et al.*, 2020). Environmental DNA (eDNA) is a developing technology that identifies species presence from the genetic material lost to their surroundings (Ficetola *et al.*, 2008). Environmental DNA often outperforms capture-based techniques (e.g., electrofishing and netting) in terms of detecting rare and elusive fish species. However, factors such as the quantity, distribution and dispersion of DNA in a water body can influence detection rates, alongside the incompleteness of reference databases, the presence of PCR inhibitors (e.g., humic acid), mutations, and DNA

degradation (Jerde *et al.*, 2011; Rees *et al.*, 2014; Evans *et al.*, 2017; Zaiko *et al.*, 2018; Harper *et al.*, 2019; Piggott *et al.*, 2021;).

Dogs have been used for conservation efforts for more than a century (Hill & Hill, 1987; Beebe *et al.*, 2016). Over this time, they have been employed to detect a variety of bird, mammal, reptile, and amphibian species (Browne *et al.*, 2006; Rolland *et al.*, 2007; Browne *et al.*, 2015; Beebe *et al.*, 2016; Matthew & Relton, 2021). A recent study investigated their potential as an early detection tool for invasive carp by assessing a single dog's sensitivity to carp-scented aquaria water and comparing its detection performance to eDNA (Collins *et al.*, 2022). The findings from this study revealed that the dog and eDNA had similar levels of sensitivity, with both methods detecting carp down to a dilution equivalent to 9.3 kg carp/ha. While these results were promising, dogs' ability to detect carp in water sourced from natural systems has yet to be investigated. This would be a significant advancement as water from natural aquatic systems likely contains highly complex odour profiles (i.e., from vegetation, other species, or sediment) compared to carbon-filtered aquaria water.

In this study, three dogs that had been trained to detect an ecologically realistic biomass of carp in aquarium water (~310 kg carp/ha) participated in two experiments where they evaluated water samples sourced from natural aquatic systems. The first experiment was a proof-of-concept experiment that assessed dogs' ability to detect a standardised biomass of carp in lake water (~310 kg carp/ha). The second experiment evaluated the generalisation[14] abilities of the dogs, by assessing their ability to detect different densities of carp in lakes of varying water quality. Environmental DNA analysis was also performed on the same samples assessed by the dogs to evaluate dogs' detection performance in relation to an existing survey technique. The use of scent detection dogs could improve conservationists' and fishery managers' ability

[14] Olfactory generalisation refers to the dog's ability to respond to similar odours in the same way as they would as the originally trained odour (Moser *et al*., 2019).

to detect early incursions of invasive carp, and thus their ability to implement control actions before the species causes significant ecological impacts.

4.3 Materials and Methods

4.3.1 Subjects

Pet dogs were recruited for this project via word of mouth, social media, and posters. Owners who expressed an interest in having their dog participate in the study were sent an initial enquiry form (refer to Appendix C, section 1 for the initial enquiry form). If there were no obvious disqualifying factors exhibited on the form once it was returned, the owner was asked to bring their dog in for an initial interview. In this interview the dog's behavioural (i.e., dog arousal levels around the researcher and other dogs, motivation to work for food) and physical (i.e., height) characteristics were evaluated to determine each dog's suitability for this research (refer to Appendix C, section 1 for the interview checklist). Of the 15 dogs recruited, three desexed females aged between 4 and 9 years of age met the participation criteria and took part in this study. Sabi and Harlee were Labrador retrievers, and Ruby was a Labrador retriever-border collie cross. The dogs were brought to the University of Waikato's Scent Detection Research Group facility twice per week by their owners. Each dog was held in a crate with bedding and water when not actively participating in experiments. None of the dogs were housed overnight.

All dogs were required to achieve autonomous operation of an automated scent detection apparatus (see Edwards 2019) and accurate detection of carp at a dilution of aquaria water equivalent to ~310 kg carp/ha before participation in the experiment. This dilution was selected as it fell within a range of carp biomasses reported for lakes in the lower Waikato region of New Zealand (Collier & Grainger, 2015; Hicks *et al.*, 2015; Tempero *et al.*, 2019). The dogs were trained to operate the apparatus and detect carp using shaping, i.e., the method of reinforcing successive approximations toward the desired behaviour (Skinner, 1975). For

more details on the training procedure refer to Collins *et al.* (2022). All experimental procedures used in this study were approved by the University of Waikato Animal Ethics Committee (protocol #1013).

4.3.2 Water Sample Collection

Water samples were collected from aquaria located at the University of Waikato's Aquatic Research Centre, or from natural lakes within the Waikato and Bay of Plenty regions of New Zealand. Two polyethylene tanks were used as aquaria. One tank (195 L) contained a single adult carp (target scent) which was replaced monthly, while the other tank (57 L) contained no fish and acted as a control. During non-experimental periods, both tanks received a continuous supply of oxygenated, de-chlorinated municipal water. All tanks had their water supply turned off 24 hours prior to collecting the water samples to concentrate carp scent; each tank was then emptied, cleaned with a designated cleaning pad, and refilled with water. To standardise sample potency, the carp tank was filled to a level where fish biomass was equal to 15.5 g/L, while the control tank was maintained on flow-through. After the 24-hour period, water was collected from each tank using an acid-washed (67% HNO_3) glass beaker and placed into species-specific acid-washed (10% HCl) Nalgene bottles. Once transported to the scent detection laboratory all samples were held in the refrigerator (4°C) until they were required for use in sample preparation. No samples were held in the refrigerator for >1 hour.

Lake water samples were collected from lakes with and without carp, covering a variety of trophic states (Table 4.1). Sub-surface water samples were collected from the shore or, when possible, from the middle of the lake using acid-washed Nalgene bottles and transported to the laboratory on ice. None of the samples were held on ice for more than 5 hours. The samples were then immediately frozen at -18°C until needed for experimental testing.

Table 4.1: Details of lakes assessed by dogs and used for eDNA analysis. Information on the lakes geomorphic type and trophic state was sourced from Hamill & Lew (2006) while the information on the presence/absence of carp was retrieved from Collier & Grainger (2015).

Lake	Geomorphic type	Trophic status	Carp present (✓) & absent (-)
Taupō	Volcanic	Oligotrophic	-
Rotoroa	Peat	Eutrophic	-
Rotoehu	Volcanic	Eutrophic	-
Rotoiti	Volcanic	Mesotrophic	-
Waahi	Riverine	Supertrophic	✓
Whangape	Riverine	Hypertrophic	✓

4.3.3 Experimental Apparatus

The automated scent detection apparatus allowed the dog to work without a handler being present in the room, mitigating potential issues associated with human cueing and subjectivity. A full description of the experimental apparatus can be found in Edwards (2019). Briefly, water samples were placed in 17 individual segments on a 1 m diameter stainless steel plate, which rotated on a carousel. A stainless-steel lid was placed on top of the segments to create individual odour chambers. The front of each segment had a hinged metal flap. When the dog placed its nose through a 10 cm opening (port) at the front of the apparatus and applied pressure to the segment flap, the flap opened, and the dog was able to assess the sample. When the dog removed its nose, the flap closed, and the odour chamber resealed. Infrared beams behind the port were broken when the dog inserted its nose, producing an audible 'beep' sound. The duration of sample assessment (i.e., infrared beam break duration) was recorded on a computer outside the experimental room.

An infrared beam break of 0.5 s was treated as an "observation," after which an omnidirectional lever on the right side of the apparatus was activated by the dog. Pressing the lever rotated the carousel and presented the dog with the next water sample. An infrared beam break of more than 4 s was treated as an "indication." If the target sample (carp) was present and the dog assessed the sample for more than 4 s (i.e., the dog "indicated"), a "hit" was

recorded and the dog was provided with reinforcement (kibble) via a wireless feeder positioned 2 m from the apparatus. A "miss" was recorded when the dog assessed a target sample for less than 4 s before advancing to the next sample. If the dog advanced to the next sample without performing an indication response to a non-target sample, a "correct rejection" was recorded. In contrast, if the dog indicated on a non-target sample, a "false alarm" was recorded. Only hits were reinforced with food (i.e., kibble).

4.3.4 Experimental Procedure

4.3.4.1 Experiment One

Experiment One examined whether dogs could detect a standardised carp biomass of ~310 kg carp/ha in water from a lake, and if their detection performance were impaired by factors such as total dissolved solids, suspended solids or odours present from other non-target fish species. Lake water samples were thawed 24 hours prior to assessment by the dogs, and no water samples were frozen for >8 weeks. Sample preparation involved filling 17 single-use polypropylene containers with 100 mL of water collected from a carp-absent lake (i.e., Taupō, Rotoroa, or Rotoehu). Seven of these samples had carp aquarium water added to them to achieve a final biomass concentration of 15 mg carp/L, which corresponds to an environmental biomass of ~310 kg carp/ha assuming a 1 ha water body with a depth of 2 m. The remaining 10 containers had fish-free (control) aquarium water added to them to ensure that the dogs were not cueing on aquaria-related scents. Once the samples were individually placed in apparatus segments, a lid was placed on top, and the samples were allowed to equilibrate with the segment headspace for 20 minutes. Each dog was then brought into the experimental room to complete a session where they assessed all 17 samples. Depending on the dog's motivation to work, each dog completed 4-6 sessions per day with a 5-minute break between sessions. Owing to the time requirements to wash and dry the apparatus components, re-randomisation of the samples after

every session was not possible. To help mitigate the potential impact of order or learning effects, all dogs performed two (Ruby) or three (Sabi and Harlee) sessions with the carousel rotating in a clockwise direction, and then two (Ruby) or three (Sabi and Harlee) sessions with the carousel rotating in an anticlockwise direction. Once the dogs had completed their sessions, the segments and samples were removed, and the apparatus was cleaned following the procedures outlined in Collins *et al.* (2022).

The dogs assessed water samples from three carp-free lakes that varied in trophic state: Lake Taupō, which is classified as oligotrophic due to its low levels of nutrient and algal productivity, and Lakes Rotoroa and Rotoehu which are eutrophic due to their high nutrient and algal concentrations. If a dog's daily average hit rate on the target (carp scent) and correct rejection rate on non-target (no carp scent) samples was ≥70% for two consecutive days (hereafter, accuracy criteria), their detection performance was considered stable, and they progressed to the next lake. A maximum exposure time of four weeks per lake (i.e., 8 experimental days) was used to aid progression of the experiment.

Due to COVID-19 related restrictions, experimental testing was halted for 4-weeks during the trials on Lake Rotoehu. Sabi and Harlee had met the accuracy criteria on all three lakes when this occurred, but Ruby was one day short of meeting the criteria. Due to the length of the break in testing and based on dogs' past performance following interruptions, it was assumed that the dogs' detection performances would have regressed when trials resumed. Therefore, to ensure that all dogs were performing satisfactorily before they progressed to the more complex experiment two, the dogs were presented with water samples from Lake Rotoehu a second time and had to meet additional accuracy criteria of average hit and correct rejection rates ≥70% for one day.

4.3.4.2 Experiment Two

Experiment Two examined whether dogs could generalise carp scent by exposing them to water samples from familiar and novel lakes that varied in trophic status and carp density (Table 4.1). As in Experiment One, lake water samples were defrosted at 21°C 24 hours prior to assessment and no samples were frozen for >8 weeks. Ten 100 mL water samples from carp-free lakes Rotoroa (n = 5) and Taupō (n = 5) were prepared. Two of the five samples allocated to each lake had carp aquarium water added to them to achieve a standard carp biomass of ~310 kg/ha. The remaining six samples (i.e., three from each lake) had water from the no-fish aquarium added to them to ensure the dogs were not cueing on aquaria related scents. The samples from Lake Rotoroa and Taupō were used to assess the dogs' baseline performance (hereafter referred to as Baseline lakes), as the dogs had been exposed to these lakes and achieved the highest accuracy with samples from these lakes during Experiment One. The remaining seven novel samples were used to determine whether the dogs responded to novel odours in the same way they would respond to a familiar odour (i.e., stimulus generalisation). Four of these seven samples contained 100 mL of water from two lakes containing naturalised populations of carp (Whangape: n = 2; Waahi: n = 2; unknown carp density), and the three other samples contained 100 mL of water from Lake Rotoiti which contained no carp. The dogs were presented with water from three novel lakes to ensure that they were not responding to an anomalous odour (Lazarowski *et al.* 2020). Sample evaluation by the dogs followed the same protocol as Experiment One, except that all dogs only performed four sessions (two sessions with the apparatus moving in a clockwise direction, and two in an anticlockwise direction). Due to a second COVID-19-related shutdown period of ~2 months, all experimental testing was halted, with each dog only completing eight sessions.

4.3.5 Environmental DNA

Environmental DNA analysis was performed on the same samples assessed by the dogs to compare the two techniques' abilities to detect carp in water sourced from lakes. Bleach (10% v/v sodium hypochlorite) cleaned glass bottles were used to collect water from the lakes, and freshwater eDNA sample kits supplied by Wilderlab (Wellington, New Zealand) were used to sample eDNA. Due to logistical issues related to obtaining carp aquarium water, lake samples that required the standardised concentration of carp water (concentration equivalent to ~310 kg carp/ha, which was the same concentration assessed by the dogs) were frozen for up to 7 days. Fresh aquaria water was then collected, added to the sample, and the sample was filtered for eDNA analysis. In contrast, samples that did not require the addition of fresh carp aquaria water were filtered within 6 hours of collection. Ultrapure water was used as a negative control. Large variations in the water quality between lakes resulted in filtered volumes ranging between 60 mL and 1 L. Filters were preserved with DNA/RNA Shield preservative (Zymo Research, California) supplied as part of the Wilderlab kits. The samples were then immediately sent to Wilderlab for analysis using both multispecies metabarcoding and species-specific qPCR tests that had been optimised for New Zealand species, and carp (*C. rubrofuscus*), respectively (refer Table 4.2 for details on the carp species-specific assay). Replicate samples were extracted from certain lakes to analyse eDNA efficiency. Equipment and budgetary constraints prevented the researcher from collecting replicate samples at all lake tested.

Table 4.2: Details of the assay used in the qPCR master mix for the testing of carp DNA samples, targeting a 186 bp fragment of mitochondrial DNA in the 16S gene region. From: Wilkinson pers. comm.

Assay	Label	Sequence (5'-3')	Fragment length (bp)
Species–specific assay: Koi carp (*Cyprinus rubrofuscus*)	*C. rubrofuscus* 16s-F	CGTGCAGAAGCGGGTATAATAC	186
	C. rubrofuscus 16s-R	GGTTTTAGGAGGTTTTCCCA	
	C. rubrofuscus 16s-Probe	AAGCAAAAACCTTGTGGACCATG AGATT (5'-/56FAM/ZEN/3IABkFQ/-3')	
	C. rubrofuscus 16s-Amplicon	CGTGCAGAAGCGGGTATAATA CAAGCAAAAACCTTGTGGACC ATGAGATTTGGGAAAACCTCCT AAAACCCGTGCAGAAGCGGGT ATAATACTACAAGACGAGAAG ACCCTTTGGAGCTTAAGGTACA AAACTCAACCACGTTAAGCAA CTCAATAAAAAGCAAAAACCT TGTGGACCATGAGATTTTACCT TCGGTTGGGGCGACCACGGAG GAAAGAAAAGCCTCCAGGTGG ACTGGGAAAACCTCCTAAAAC C CGTGCAGAGGCGGGTATAATA CTACAAGACGAGAAGACCCTT TGGAGCTTAAGGTACAAAACT CAACCACGTTAAGCAACTCAA TAAAAAGCAAAAACCTTGTGG ACCATGAGATTTTACCTTCGGT TGGGGCGACCACGGAGGAAAG AAAAGCCTCCAGGTGGACTGG GAAAACCTCCTAAAACC	

4.3.6 Total Suspended Solids, Conductivity, and Total Dissolved Solids

The total suspended solid content, conductivity and concentration of total dissolved solids in each lake sample were measured. This was to determine if water quality impacted dogs' detection performance as samples with a higher algal or nutrient content may contain a greater number of odours that could affect dogs' ability to detect carp scent. For total suspended solids analysis, lake water samples were vacuum-filtered through ashed and pre-weighed glass fibre filters (GC50, Advantec). The samples were then dried at 100°C for 24 hours and re-weighed

to determine the total suspended solids. The filters were then combusted at 550°C for 1 hour and weighed again to determine inorganic suspended solids (ISS) and volatile suspended solids (VSS). A YSI EC300 conductivity meter was used to measure the conductivity and total dissolved solids.

4.3.7 Statistical Analysis

4.3.7.1 Experiment One

Two accuracy measures were calculated using the dogs' pre-COVID-19 shutdown data to determine whether dogs could accurately detect a commonly occurring biomass of carp in lake water, and whether the lakes' trophic state affected the ability of dogs to detect carp. Dogs' sensitivity to carp was determined by dividing correct positive indications by the sum of correct positive- and missed-indications, while their specificity was determined by dividing correct rejections by the total number of correct rejections and false alarms (Wong & Lim, 2011).

COVID-19 restrictions forced the dogs to take a four-week break during experimental testing. To determine if this break affected the dogs' detection performance, the Lake Rotoehu data were separated into two groups: before and after the COVID-19 shutdown. Sensitivity and specificity values were then calculated for each group.

Sample rearrangement after each session was not possible; as such, there was concern that repeated exposure to a sample array may have influenced the detection performance of dogs. To evaluate this concern, sensitivity and specificity values were calculated for each session performed by the dogs.

Inferential statistics were considered for the data, including Friedman's non-parametric test (common biomass data), non-parametric t-test (before and after COVID-19 shutdown data), and Generalised Linear Mixed Model (session data). However, the small sample size (i.e., three dogs) would have resulted in low statistical power, increasing the chance of a type II error.

Consequently, it was determined that descriptive statistics and visual analysis of data were more appropriate for these data.

4.3.7.2 Experiment Two

Sensitivity and specificity associated with each lake were calculated and 95% confidence intervals were applied to the means. To determine if a dog was performing above chance levels on baseline and novel lake samples, the total number of indications was multiplied by the percentage of samples of each type. For example, if a dog indicated 10 of 17 samples as positive (58.8% indicated) and positive samples made up seven out of 17 samples, the number of correct positive indications expected by chance would be 58.8% of seven, or approximately 4 (4.12). The same approach was taken with negative samples. A dog was considered to have performed above chance levels if the value at the lower 95% confidence interval limit was greater than the calculated value of expected chance performance. The limited sample size for each lake precluded the statistical testing of dog scent detection performance between lakes. All graphs and descriptive statistics were completed or performed using GraphPad Prism (Version 9).

4.4 Results

4.4.1 Experiment One

The dogs took an average of 24.7 sessions to meet the accuracy criteria when presented with lake water sample containing a standardised biomass of carp and took on average 192 s to complete a session of 17 individual sample assessments (Table 4.3).

Table 4.3: Mean number of sessions and time (± 95% confidence interval) the dogs (n = 3) took to complete a session for each lake examined both before and after the COVID-19 shutdown.

Lake	Mean session (n)	Mean session time (s)
Taupō	21.3 ± 11.5	192 ± 12.6
Rotoroa	32.0 ± 17.2	175 ± 11.7
Rotoehu (before shutdown)	24.0 ± 29.8	190 ± 14.3
Rotoehu (after shutdown)	21.3 ± 11.5	212 ± 14.6
Mean	24.7 ± 5.1	192 ± 24.7

Figure 4.1 illustrates that dogs' sensitivity and specificity to a standardised biomass of carp did not change considerably across lakes (i.e., Taupō, Rotoroa, Rotoehu). Assessment of the dogs' data before the COVID-19 lockdown revealed that the dogs' mean sensitivity values remained ≥83.6% and specificity values remained ≥77.3% on all lakes tested.

Comparisons of dogs' detection performance on Lake Rotoehu samples before and after the 4-week break revealed that the dogs' mean sensitivity decreased from 83.6% to 82.2% after the break, while their mean specificity values decreased from 77.3% to 67.4% (Figure 4.1).

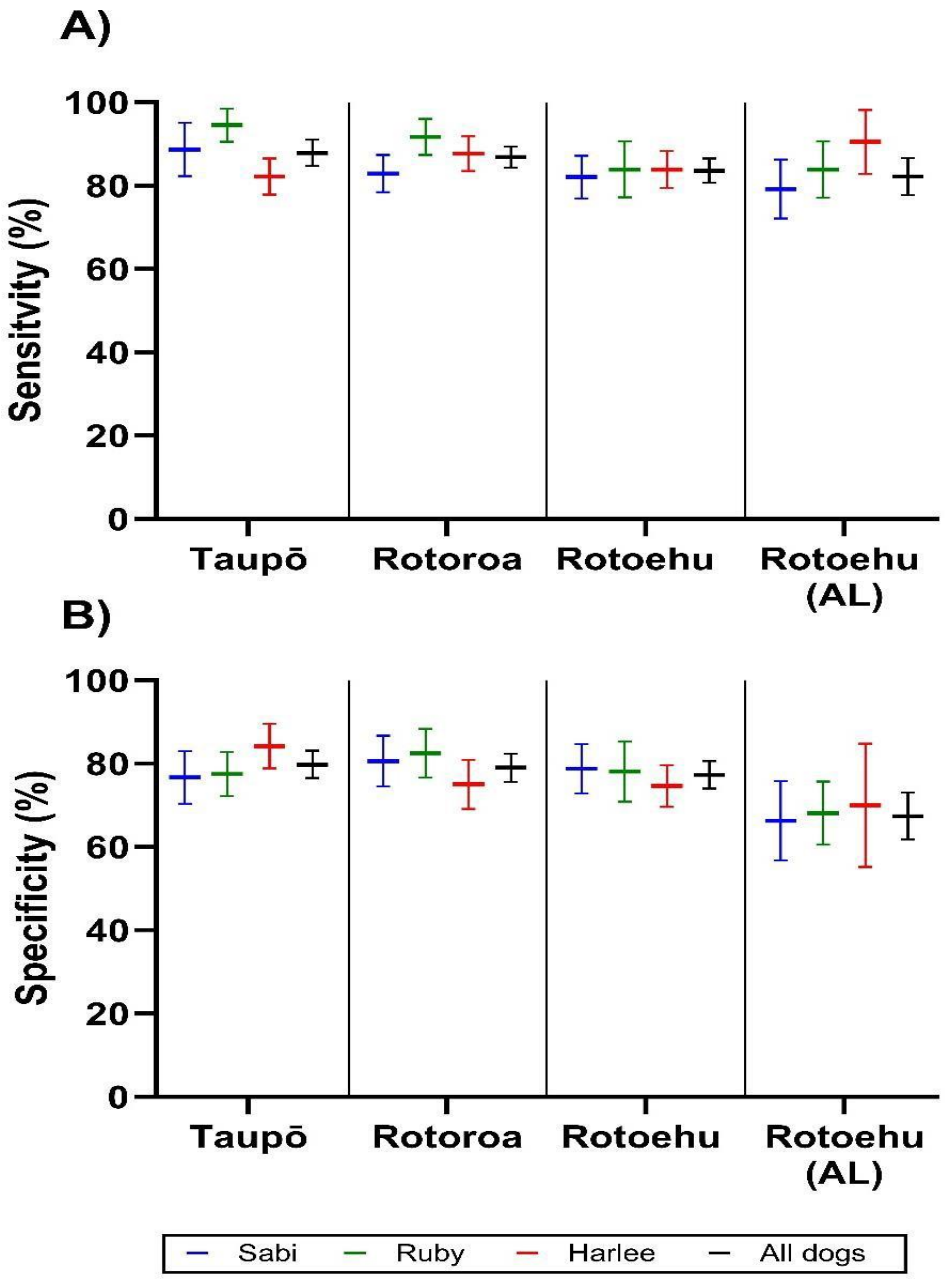

Figure 4.1: Individual and all dogs' (*n* = 3) mean (± 95% confidence interval) (A) sensitivity and (B) specificity values for each lake, tested before (Taupō, Rotoroa, Rotoehu) and after (Rotoehu (AL)) the COVID-19 lockdown.

Session number had a minimal influence on dogs' sensitivity to carp (mean range regardless of session was 83.3% to 87.6%). However, it did influence dogs' specificity of carp detection. The dogs' mean specificity in session one (61.9%) was lower than in session two

(76.6%) and all other subsequent sessions (Session three = 75.0%; Session four = 76.6%; Session five = 80.4%; Session six = 83.0%; Figure 4.2).

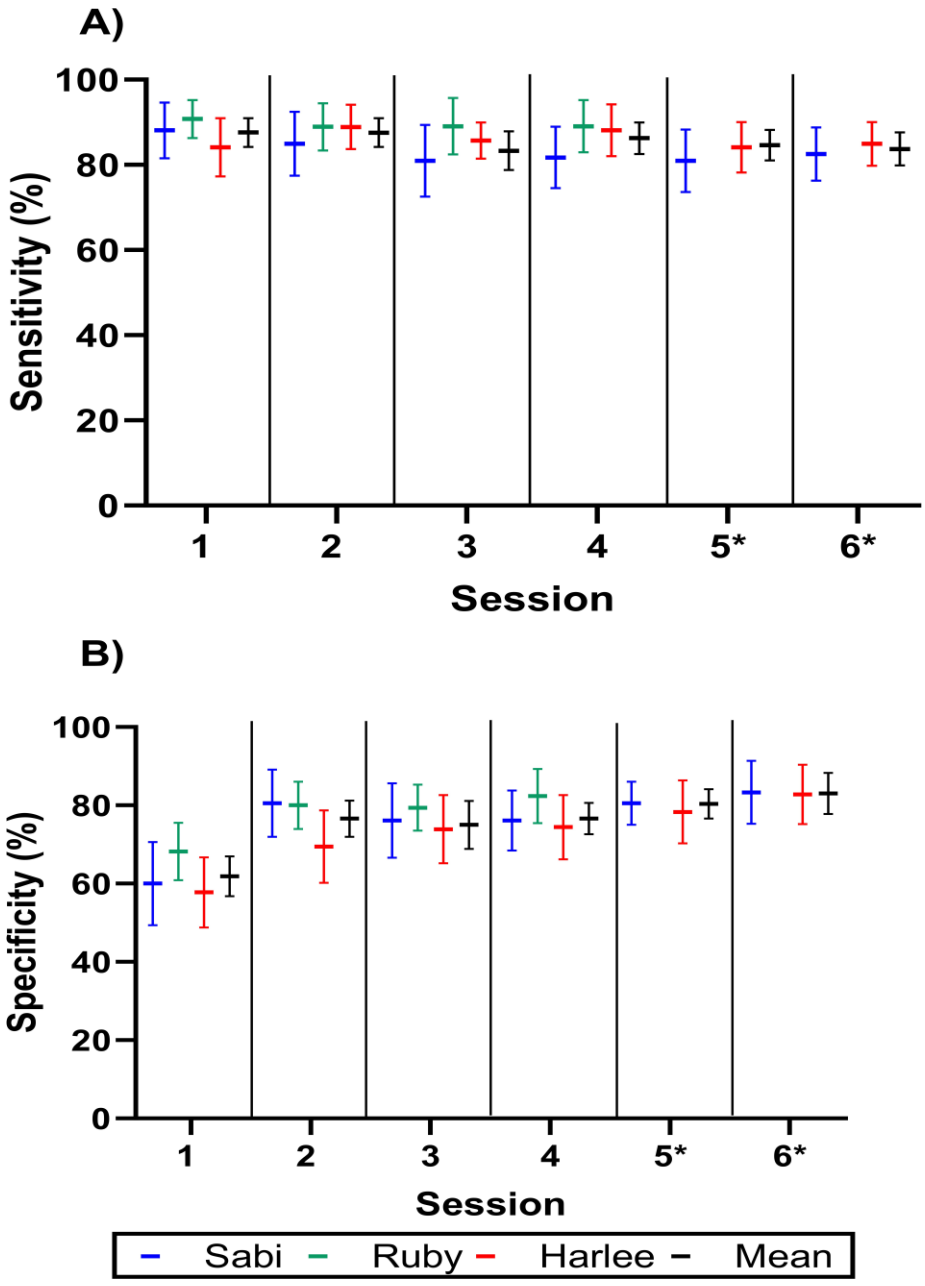

Figure 4.2: Individual and all dogs' (*n* = 3) (A) mean sensitivity on carp and (B) mean specificity (± 95% confidence interval) in each session. These data are summarised from all lakes tested in Experiment One. * Indicates sessions where Ruby did not participate.

4.4.2 Experiment Two

All dogs completed eight sessions. The mean performance of dogs revealed that the sensitivity of dogs to novel lake water samples (Whangape = 100%, Waahi = 97.9%) was higher than their sensitivity to lake water with which they had prior exposure (i.e., baseline samples; Rotoroa + carp = 52.1%, Taupō + carp = 66.7%). A similar trend was observed for the dogs' specificity; the dogs' specificity was higher for the novel carp-absent lake (Rotoiti = 70.1%) than for the carp-absent lakes they had been exposed to in Experiment One (Rotoroa + no fish = 62.5%; Taupō + no fish = 55.5%; Figure 4.3).

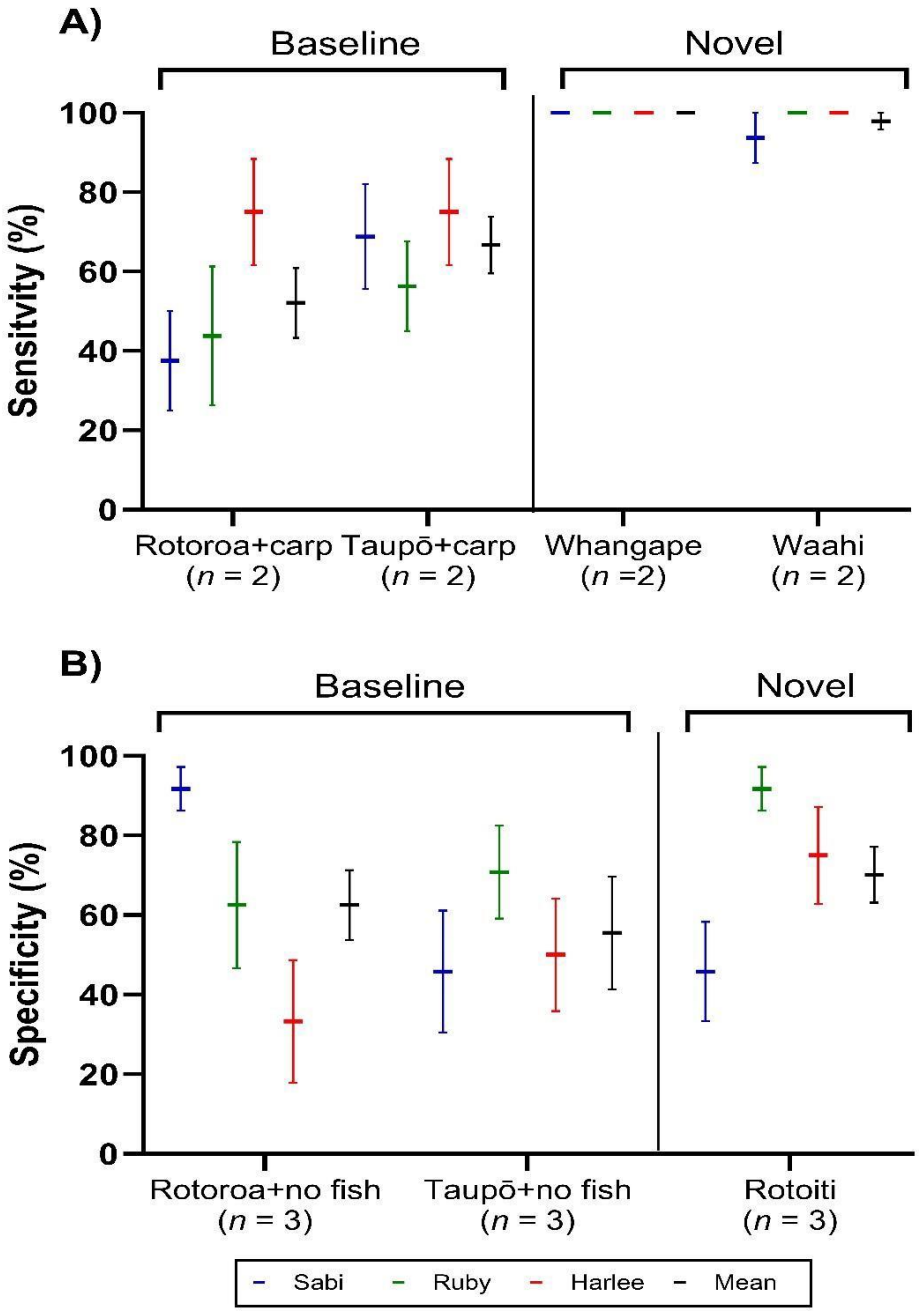

Figure 4.3: Individual and all dogs' mean (A) sensitivity and (B) specificity (± 95% confidence intervals) values for baseline and novel lake samples. '*n*' = the number of samples allocated to each sample type per sample array (total 17 samples).

The dogs' mean percentage of correct responses (± 95% confidence intervals) and their chance performance rates on baseline lakes (baseline + carp and baseline + no carp) and novel lakes (novel carp and novel no carp) is displayed in Figure 4.4. Although no statistical analysis was performed due to the low sample numbers, the overlap between the chance performance

threshold and the 95% confidence interval for mean correct responses indicates that the dogs may not have been performing better than chance on baseline + carp samples. Sabi and Ruby both performed above chance on baseline samples containing water from a fish free aquarium, but Harlee was well below the chance threshold. All dogs performed above chance on novel samples collected from lakes containing naturalised populations of carp, while Ruby was the only dog to perform above chance on novel samples collected from a carp free lake.

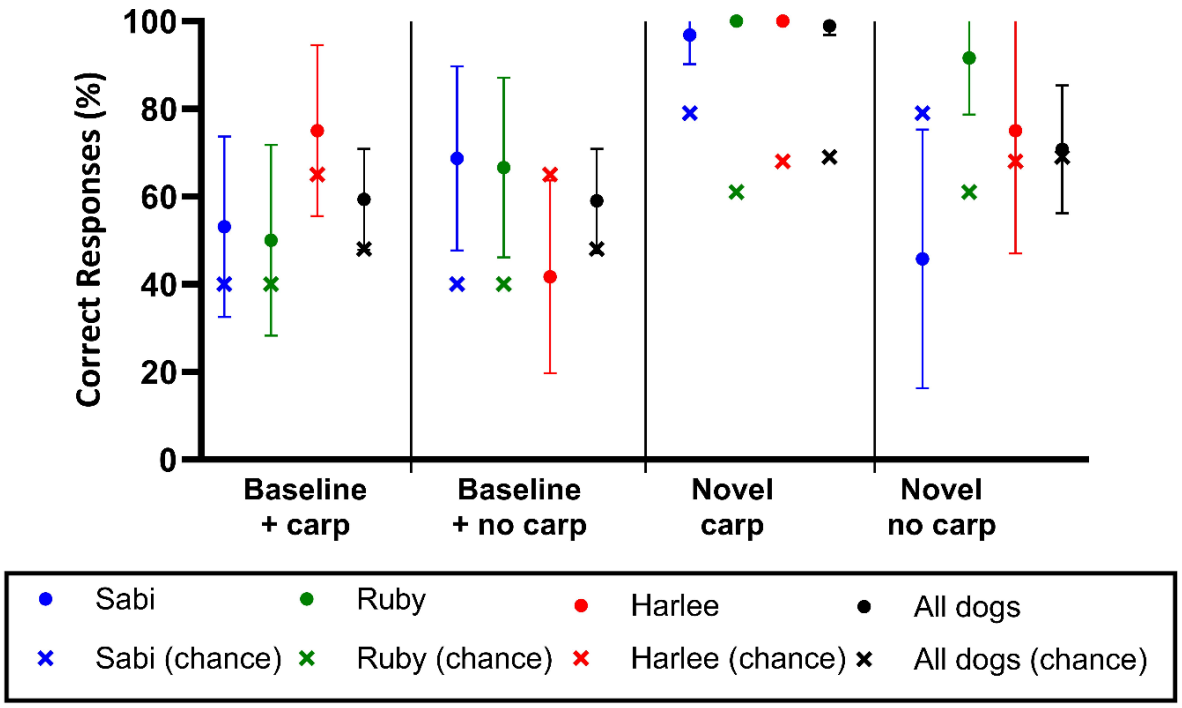

Figure 4.4. Dogs' mean percentage of correct responses (± 95% confidence intervals) and their chance performance rates on baseline lakes (baseline + carp and baseline + no carp) and novel lakes (novel carp and novel no carp).

4.4.3 Environmental DNA

Table 4.4 presents the total DNA reads detected in the samples for all taxonomic groups (i.e., bacteria, aquatic invertebrates, birds and terrestrial species), and the number of reads assigned to the fish species. The results from the qPCR method are also presented. Multispecies metabarcoding successfully detected carp in three out of the five samples known to contain

carp scent. Where duplicate samples were taken, it was evident that certain species were detected in one, but not the other eDNA sample. For example, goldfish was detected at the outlet at Lake Rotoroa, but not at the ramp in Lake Rotoroa. The employment of the single-species qPCR method resulted in the detection of carp in all, but one lake known to contain naturalised populations of carp (i.e., Lake Waahi). No correlation between qPCR CT value and relative carp biomass was found.

Table 4.4: Environmental DNA results for water samples collected from natural lakes employing multispecies metabarcoding and single-species qPCR. Baseline samples were from lakes where the dogs had achieved the highest accuracy in Experiment One and had either no carp aquaria water added to them (i.e., baseline lakes + no carp) or a concentration of carp aquaria water equivalent to~310 kg carp/ha added to them (i.e., baseline lakes + carp). Novel samples were collected from lakes to which the dogs had no prior exposure and contained no carp (i.e., novel lake-carp absent) or naturalised populations of carp (novel lakes-carp present). Only lakes that required the addition of carp aquarium water were frozen (Fr) before eDNA extraction (frozen for a maximum of 7 days); all other samples had their eDNA extracted within 6 hours of collection (fresh = F). Indicative biomass values were calculated from the fish biomass water used to spike the samples. X = species present but not detected by eDNA; ✓ = species correctly identified as present; - = species correctly identified as absent. Numbers in parentheses are the number of successful qPCR amplifications, e.g., 1/4, and NA indicates no PCR amplification. [15]

Species		Baseline lakes + no carp										Novel lake - carp absent		Baseline lakes + carp						Novel lakes - carp present			
	Negative Control	Taupō (F)		Rotoroa (outlet; F)		Rotoroa (ramp; F)		Rotoehu (F)		Rotoehu (F)		Rotoiti (F)		Taupō (Fr)		Rotoroa (Fr)		Rotoehu (Fr)		Waahi (F)		Whangape (F)	
Multispecies Assay																							
Carp (*C. rubrofuscus*)	0 -	0	-	0	-	0	-	0	-	0	-	0	-	120	✓	0	x	0	x	243	✓	6	✓
Bullies (*Gobiomorphus* spp.)	0 -	554	✓	1501	-	2096	✓	1195	✓	733	✓	0	x	1222	✓	0	x	311	✓	112	✓	559	✓
Catfish (*Ameiurus nebulosus*)	0 -	0	x	42	✓	184	✓	0	-	0	-	0	x	0	x	0	x	0	-	0	x	0	x
Goldfish (*Carassius auratus*)	0 -	0	x	0	x	67	✓	501	✓	365	✓	0	x	0	x	0	x	172	✓	211	✓	0	x
Smelt (*Retropinna retropinna*)	0 -	288	✓	0	-	0	-	278	✓	216	✓	0	x	14	✓	0	-	0	x	0	x	0	x
Koaro (*Galaxias brevipinnis*)	0 -	148	✓	0	-	0	-	0	-	0	-	0	x	0	x	0	-	0	-	0	x	0	-
Rainbow Trout (*Oncorhynchus mykiss*)	0 -	0	x	0	-	0	-	0	x	38	✓	0	x	0	x	0	-	0	x	0	-	0	-
Mosquito fish (*Gambusia affinis*)	0 -	0	-	0	x	130	✓	0	-	0	-	0	-	0	-	0	x	0	-	0	x	0	x
Perch (*Perca fluviatilis*)	0 -	0	-	0	x	0	x	0	-	0	-	0	-	0	-	0	x	0	-	622	✓	0	x
Tench (*Tinca tinca*)	0 -	0	-	37	✓	0	x	0	-	0	-	0	-	0	-	0	x	0	-	0	-	0	-
Shortfin eel (*Anguilla australis*)	0 -	0	-	0	x	0	x	0	-	0	-	0	x	0	-	0	x	0	-	0	x	90	✓
Volume filtered (mL)	1,000	800		750		920		800		800		1,000		500		500		800		250		60	
Total fish reads	0	990		1,580		2,477		1,974		1,352		0		1,356		0		483		1,188		655	
Total eDNA reads	2,124	7,639		15,768		16,917		11,897		20,567		2,612		9,082		5,565		4,419		8,262		4,707	
Single-species qPCR																							
Carp qPCR – CT value*	NA	NA		NA		NA		NA		NA		NA		36.0 (1/4)		37.0 (1/4)		35.8 (1/4)		NA		36.3 (3/4)	
Carp biomass	0	0		0		0		0		(		0		310		310		310		Unknown		Unknown	

[15] Information on presence and absence of fish are sources from Collier and Grainger (2015), West *et al.* (2000), Hayes and Rutledge (1991), Rowe (1993), Barnes and Hicks (2003), and Kusabs *et al.* (2015).

4.4.4 Suspended Solids Analysis

Table 4.5 shows the specific conductivity of the lakes, as well as the results obtained from the suspended solid analysis. Total suspended solids are composed of organic particles such as decaying organic matter, bacteria, zooplankton and algae. Inorganic suspended solids are the mineral based fraction. Lakes containing naturalised populations of carp had a higher suspended solid content than lakes with no carp (Table 4.5).

Table 4.5: Lake parameters and results from suspended solid analysis of water samples. Bracketed values indicate the percentage of the total suspended solids.

Lake	Specific conductivity (mS/cm)	Total dissolved solids (ppt)	Total suspended solids (g/m^3)	Volatile organic solids (g/m^3)	Inorganic suspended solids (g/m^3)	Carp present (✓) & absent (-)
Experiment One						
Taupō	0.16	0.10	0.7	0.3 (43%)	0.4 (57%)	-
Rotoroa	0.13	0.09	5.7	2.4 (42%)	3.3 (58%)	-
Rotoehu	0.64	0.41	4.3	1.3 (30%)	3.0 (70%)	-
Experiment Two						
Taupō	0.16	0.10	0.8	0.1 (12%)	0.7 (88%)	-
Rotoroa	0.13	0.08	9.6	5.4 (56%)	4.2 (44%)	-
Rotoiti	0.16	0.13	4.5	3.2 (71%)	1.3 (29%)	-
Waahi	0.33	0.22	31.2	5.4 (17%)	25.8 (83%)	✓
Whangape	0.21	0.14	218.2	31.8 (15%)	186.4 (85%)	✓

4.5 Discussion

Dogs' ability to detect the scent of carp in water sourced from natural lakes was examined to evaluate their potential for invasive fish detection. Results from Experiment One indicated that dogs could detect a standard biomass of carp (~310 kg carp/ha) in lakes of various trophic states, achieving sensitivity values of >83.6% and specificity values of >77.3% on all tested lakes. When task complexity increased and the dogs' assessed samples from lakes that varied in both carp biomass and water quality (i.e., Experiment Two), their detection performance decreased, and in some instances, failed to exceed chance performance. Despite this suggesting that dogs' may not be able to generalise carp scent, COVID-19 related disruptions severely restricted the sample size for Experiment Two. Other studies have demonstrated that dogs can detect target organisms in complex environments, sometimes achieving higher detection rates than existing survey techniques (Smith *et al.*, 2001; Grimm-Seyfarth *et al.*, 2019; Grimm-Seyfarth *et al.*, 2021). Thus, it is possible that with additional training or testing, dogs may be able to detect different biomasses of carp in a range of water qualities.

Environmental DNA was also susceptible to detection error, with the multispecies assay only detecting carp in three of the five lake samples known to either contain carp populations or carp-scented aquarium water. Quantitative PCR improved the overall detection rate to four out of five, but failed to detect carp in Lake Waahi, even though a positive carp detection was reported using the multispecies assay. These false negative detections could be due to a range of factors including lack of DNA in the sample, naturally occurring substances inhibiting PCR reactions (i.e., humic acid), or insufficient primer sensitivity (Rees *et al*., 2014; Evans *et al*., 2017). They could also have resulted from errors or omissions in the sample collection process. Specifically, freezing and thawing the spiked water samples prior to filtration may have degraded the DNA and reduced detection rates (Takahara *et al.*, 2015; Williams *et al.*, 2016). If dogs prove successful at detecting carp in naturally sourced water, they could be used as a

standalone, or complementary method of invasive fish detection. Using dogs and eDNA together could reduce the chance of error and improve the reliability of the detection results.

Session number was associated with no notable change in the dogs' sensitivity to carp, but a moderate increase in the dogs' specificity was observed after the first session. Three potential explanations exist for this change in performance. First, logistical constraints prevented sample rearrangement after each session; therefore, the dogs may have learnt the order of the samples in the first session, resulting in increased specificity in all subsequent sessions. In an attempt to prevent the dogs from learning the sample order the direction of sample presentation was reversed every 2–3 sessions and the dogs were provided 5-minute breaks between sessions. However, the effectiveness of these steps in preventing order learning cannot be determined without further research. Second, dogs were required to indicate longer on target samples than non-target samples. Consequently, odour chambers containing target samples may have ended up with a greater concentration of dog-related volatile organic compounds (VOCs) than chambers containing non-target samples. Given that the equipment was not cleaned between sessions or dogs, it is possible that the dogs used their own VOCs, or another dog's VOCs to influence their detection decision (Ferry *et al.,* 2019). Finally, it is possible that the volatiles released from the sample did not reach equilibrium with the segment headspace until the second session. The concentration of VOCs increases to a point of equilibrium in enclosed spaces (Lazarowski *et al.*, 2020). Thus, a lower concentration of VOCs may have been present in the first session, making it harder for dogs to distinguish between target and non-target odours.

Evaluation of dog performance before and after a 4-week interruption revealed a 1.9% decrease in mean sensitivity, and a 9.9% decrease in mean specificity of carp scent detection, indicating that the break had a minor impact on the dogs' detection performance. This was similar to the findings of Rutter (2021), where there was no statistical difference in dogs' ability

to detect myrrh oil after a 14-week hiatus. However, the authors noted that there was a slight decrease in the dogs' performance on odour-absent tasks once the dog returned to work. Given that there has been limited research into the impact of extended breaks on dogs' detection performance, we recommend that dogs' detection accuracy should always be evaluated after a longer than normal break. If accuracy is maintained, experimental testing can resume immediately. However, if there is a decrease in the dogs' detection performance, then remedial training should be performed until the desired level of accuracy is regained.

Interestingly, Experiment Two revealed that the dogs performed better on novel lakes (i.e., Whangape and Waahi) containing carp than familiar lakes that had been spiked with a set concentration of carp scent (i.e., Taupō and Rotoroa; 310 kg carp/ha). A potential explanation for this trend in responding is that the novel lakes may have contained a greater carp biomass than the familiar lakes. The current fish biomass in Lakes Waahi and Whangape is unknown. Single-pass electrofishing surveys conducted between 2007 and 2014 estimated Lake Waahi to contain 22.2 kg carp/ha and Lake Whangape to contain 98.8 kg carp/ha (Hicks *et al.*, 2015). However, this was likely an underestimation as single-pass electrofishing typically only catches ~48% of the total population (Hicks *et al.*, 2015). Moreover, it is possible that the carp populations within these lakes have increased since these surveys were performed, potentially exceeding the biomass found in familiar lake samples. The suspended solid content within the samples and the reinforcement schedule for this experiment could provide an alternate explanation for the superior performance on novel lakes containing naturalised populations of carp. Novel samples that contained naturalised populations of carp had a higher suspended solid content than familiar lake samples that had been spiked with carp scent (Table 4.5). Because correct responses to these samples were reinforced and the dogs were repeatedly exposed to these samples, each dog could have learnt to associate reinforcement with the scent released from the sediment and not the scent released from carp themselves. Filtering the water

samples before presentation to dogs would standardise suspended solid content and thus could potentially mitigate this issue. However, this strategy would be labour intensive, and it would increase the risk of cross contamination due to increased handling of the samples.

Pet dogs were used in this study as: (1) they did not need to be housed at the scent detection facility, reducing costs, and (2) they spent non-working periods at home in an enriched environment, instead of a kennel. However, despite these advantages, there were limitations with the pet dog approach. For example, most owners could only bring their dog into the laboratory twice per week, limiting the frequency of training and testing sessions. Although the researcher regularly communicated with owners about their dogs' weight, feeding schedule and any significant events, they had limited control over the dogs' feeding or exercise regimes. Consequently, there were occasions where the dog came into the laboratory over- or under-satiated or fatigued. Based on the findings of other studies, over-exercising (Gazit & Terkel, 2003) or under-feeding a dog (Miller & Bender, 2012) prior to experimental testing can influence the dog's motivation to work, and negatively impact its detection performance.

Replication is one of the most critical aspects of eDNA sampling. Given that DNA is not uniformly distributed within a waterbody, it is important to extract several samples to reduce the likelihood of false negative errors (Dickie *et al*., 2018; Stauffer *et al*., 2021). The number of samples taken from a waterbody is dependent on the question being asked as well as the waterbody's size (Dickie *et al*., 2018; Stauffer *et al*., 2021). However, it is recommended that a minimum of six replicates per sample site is taken to give optimal detection rates for fish (Melchior & Baker 2023). In this study, resource constraints meant that a maximum of two samples per lake could only be collected. Consequently, this may have reduced reliability of our eDNA results.

4.5.1 Conclusion

This study has demonstrated that dogs are capable of detecting an ecologically realistic biomass of carp in lake water. However, further investigations are necessary to determine the extent of their generalisation abilities. Dogs must be able to detect different densities of carp across a range of water qualities to be an effective detection method. If laboratory-based dogs prove successful in generalising carp scent, they could provide researchers with a rapid and efficient tool for invasive fish detection.

4.6 References

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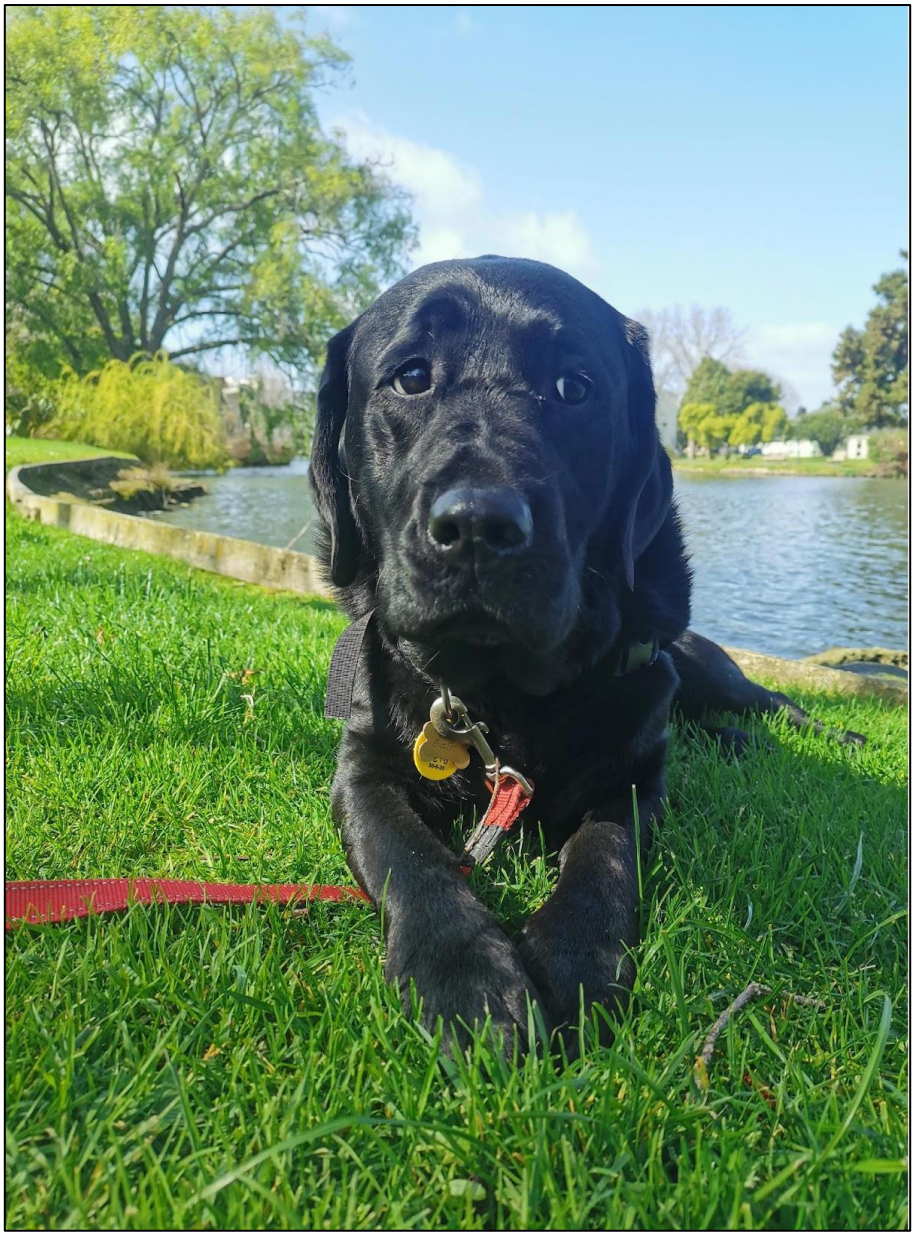
Chapter 5

General Discussion



Scent detection dog Sabi

5.1 Overview

Carp pose a significant threat to freshwater ecosystems worldwide through their high fecundity and destructive feeding behaviour (McDowall, 1996; Hicks & Ling, 2015; Weber & Brown, 2009; Bajer & Sorensen, 2015; Pietsch & Hirsch, 2015; Qiu *et al.*, 2019). Once carp reach a critical biomass, ecosystem regime shifts can occur where clear, macrophyte dominant waterbodies change to turbid, phytoplankton dominant systems (Hanchet, 1990; Roberts *et al.*, 1995; Bajer *et al.*, 2009; Weber & Brown, 2009; Collier & Grainger, 2015; Pietsch & Hirsch, 2015). Early detection and rapid responses increase the likelihood of containing or eradicating invasive fish before they establish populations and cause significant ecological impacts. However, current invasive fish detection techniques (e.g., eDNA, electrofishing, netting, or visual survey) are often expensive, time consuming and/or lack sensitivity at low fish densities (Jerde *et al.*, 2011; Hicks *et al.*, 2015ab; Lintermans, 2015; Evans *et al.*, 2017). The development of a pest fish surveillance method which improves detection efficiency at low densities could enhance managers' ability to successfully control or eradicate populations before they reach ecologically harmful densities.

Scent detection dogs have advanced olfactory systems and have proven extremely efficacious at detecting rare or cryptic species in terrestrial settings (Long *et al.*, 2007; Goodwin *et al.*, 2010; Thomas *et al.*, 2020); however, scent detection of aquatic species has not been widely investigated (Rolland *et al.*, 2007; DeShon *et al.*, 2016; Rolland *et al.*, 2017; Wasser *et al.*, 2017). The aim of this thesis was to determine whether laboratory-based scent detection dogs had the potential to detect invasive carp. This was evaluated in three experimental studies. The first study (Chapter 2) examined dogs' sensitivity and specificity to carp scent in carbon-filtered aquaria water and compared their detection performance to eDNA. This experiment was performed to determine if: (1) dogs could detect carp at low densities, similar to those that

would occur when a new invasion happens (i.e., <10 kg carp/ha), and (2) if dogs' performance was comparable to that of eDNA, one of the most sensitive invasive fish detection methods.

The second study of this thesis (Chapter 3) investigated the impact of three sample preservation techniques: refrigeration, freezing, and the addition of potassium sorbate, on dogs' detection performance to determine their potential to be used as preservatives for water samples evaluated by dogs. If laboratory-based scent detection dogs are used for carp surveillance, they would assess samples that have been collected from the field. Depending on the remoteness of the field site, it could take hours or even days for the sample to reach the laboratory and be analysed by the dogs. To maintain the samples' integrity over this time, methods that retard the biochemical (i.e., photodecomposition, thermal or microbial decomposition, or oxidation) and/or physical reactions (i.e., volatilisation) that cause scent degradation would need to be applied.

The final study of this thesis (Chapter 4) examined dogs' ability to detect carp scent from water sourced from natural aquatic systems (i.e., lakes). This is a vital step in determining dogs' viability as an early detection method, as water sourced from natural systems likely contains more background odourants than aquaria water, potentially impacting dogs' sensitivity and specificity to carp.

This concluding chapter provides a synthesis of the key findings and implications of each research chapter. It also discusses the limitations associated with this project's methodology, and future research recommendations.

5.2 Key Findings & Implications

While dogs have long been used to detect rare and elusive organisms in terrestrial environments (Hill & Hill, 1987; Hurt & Smith, 2009), Chapter 2 (i.e., Collins *et al.*, 2022) adds to the growing body of evidence that dogs can also be used to detect low-density aquatic species (Rolland *et al.*, 2007; DeShon *et al.*, 2016; Wasser *et al.*, 2017). Comparisons between dogs'

and eDNA's sensitivity to carp in aquaria-collected water samples revealed that both methods could detect carp down to a dilution equivalent to 9.3 kg/ha. Given that ecological impacts from carp typically occur at biomasses exceeding 100 kg/ha (Bajer *et al*., 2009; Vilizzi *et al*., 2014), the results of this chapter indicate that laboratory-based scent detection dogs have potential to be deployed, alongside eDNA, as an early detection method for invasive carp.

Closely-related species are thought to share a greater proportion of signature odours than distant relatives (Heth & Todrank, 2000). Consequently, discrimination between the odours of closely related species is thought to be a harder task. In addition to demonstrating dogs' acute sensitivity, Chapter 2 revealed that the detection dog had remarkable discriminative abilities. Despite carp and goldfish being close relatives that are capable of hybridising, the dog was able to discriminate the scent of the two fish species with high levels of specificity (Banks *et al*., 2010). Similar results have been reported by other studies investigating dogs' discrimination of genetically similar species. For example, Smith *et al.* (2003) revealed that dogs could discriminate kit fox (*Vulpes macrotis*) scat from red fox (*Vulpes vulpes*) scat with 100% accuracy, while Hurt *et al.* (2000) demonstrated that dogs could discriminate American black bear (*Ursus americanus*) from brown bear (*Ursus arctos*) scats with 78% accuracy. Being able to discriminate target from non-target fish species is crucial to dogs' success as a carp detection method, as false positive indications could lead to the implementation of unnecessary control actions. These could harm non-target species and cost fishery managers significant amounts of time and money.

The results from Chapter 3 also support the potential utilisation of laboratory-based dogs as an invasive carp detection method. Once the dogs had met a stable state of performance, their detection performance on frozen, or potassium sorbate preserved samples was comparable to, or better than, their detection performance on baseline samples. This indicated that these preservation methods may have effectively retarded scent degradation over the seven- or eight-

day storage period. Although additional research with a larger sample size is required, these results are promising and indicate that freezing and/or potassium sorbate could be effective methods for preserving samples during their transit to the scent detection facility.

While other studies have previously demonstrated the efficacy of freezing as a preservation method for canine assessed samples (Chi *et al.*, 2021; Needs *et al.*, 2021), Chapter 3's study is the first to demonstrate the potential use of potassium sorbate. Using potassium sorbate as a preservation technique could improve researchers or other organisation's ability to collect and store aqueous samples for scent detection. Potassium sorbate is an inexpensive, readily available and easily transportable chemical that permits storage at room temperature. Its use has the potential to compare favourably with methods such as refrigeration and freezing, which often lack portability to remote locations (Dehghan *et al.*, 2018).

After demonstrating that dogs could detect carp in aquaria-collected water samples (Chapters 2 & 3), the next step of determining dogs' potential as an invasive carp detection method was to evaluate their ability to detect carp in water sourced from natural lakes. The results from Experiment One of Chapter 4 demonstrated that dogs could accurately detect an ecologically realistic biomass of carp (i.e., 310 kg /ha) in lake water, with all dogs achieving sensitivity values >83.6% and specificity values >77.3% on lakes covering a range of trophic states. When task complexity increased in Experiment Two and the dogs evaluated samples from lakes that varied in novelty, carp biomass, and water quality, the dogs' detection performance decreased, with the dogs' mean correct responses being above chance on some, but not all lakes tested. Stimulus generalisation is important to dogs' success as a detection method as the inability to detect variations in carp scent (e.g., different biomasses of carp) and discriminate carp from non-target odours could result in false negative and false positive detections. Other research has revealed varying results, with dogs' performance generalising to some but not all target scents (Wright *et al.* 2017; Moser *et al.*, 2019). For example, Cerna *et*

al. (2011) found that dogs trained with pure trinitrotoluene (TNT, $C_7H_5N_3O_6$) did not successfully generalise to actual TNT targets, which were of different origins and varied in overall composition. In contrast, Oldenburg *et al.* (2016) revealed that a detection dog was able to generalise novel otter (*Lutra lutra*) samples after being trained with only two variations of faeces. Due to the limitations imposed by COVID-19 (i.e., early termination of Experiment Two and thus the insufficient collection of data), further research is required to determine if dogs can sufficiently generalise carp scent.

When eDNA analysis was performed on the samples assessed by the dogs, the multispecies assay detected carp in three out of five of the lake samples known to contain carp scent. Quantitative PCR using the species-specific primer increased the detection of carp, yielding positive results from four out five lakes, but still failed to detect carp in Lake Waahi, which is known to contain a naturalised carp population (Collier & Grainger, 2015). The exact reason for this detection failure is unknown but could be related to the amount, quality and distribution of DNA in the waterbody, sample volume, or the strategy used to collect, store or analyse the sample (Rees *et al.*, 2014; Rishan *et al.*, 2023). Given that dogs and eDNA were both susceptible to detection errors, they may be best used as complementary detection techniques. Using both techniques simultaneously could improve the reliability of the results and thus confidence in implementing or not implementing control actions.

In summary, these research chapters have demonstrated that dogs can detect carp in water sourced from aquaria. They have also demonstrated for the first time that potassium sorbate may have potential as a preservative for canine assessed samples, and that dogs may have similar sensitivity levels to eDNA, one of the most efficacious methods for detecting low density aquatic organisms. However, further investigations into dogs' ability detect variations of carp (i.e., different densities of carp) in naturally sourced water with a range of background odourants is required to determine if they could become a viable detection method. If dogs do

prove successful at detecting carp in naturally sourced water they could serve as an efficient method of carp detection, improving researchers' ability to survey lakes and other aquatic systems for invasive species.

5.3 Advantages and Limitations of Using Canines as Invasive Fish Detectors

If dogs became a surveillance tool for new incursions of carp, they would assess samples that have been collected from the field and brought into the laboratory. This non-invasive approach to fish detection negates the possibility of unintended by-catch and injury to the target or a non-target organism, all of which are possible with capture-based techniques (i.e., netting and electrofishing) (Hicks *et al.*, 2015b; Lake, 2013ab). Depending on the size and resources available, surveying a single lake can take hours to days using capture-based techniques or visual surveys, particularly if the target species is present at low densities (e.g., Jerde *et al.*, 2011). Environmental DNA can be much quicker; however, it can still take several hours to process a single set of samples, and the costs are still prohibitive for integration with regular, large scale survey programmes (Jerde *et al.*, 2011; Evans *et al.*, 2017). The automated scent detection apparatus would allow dogs to assess samples from multiple waterbodies within a matter of minutes (Collins *et al.*, 2022). The high sample throughput achievable with the apparatus would likely make this a cost-efficient method of invasive fish detection. In addition, netting and electrofishing surveys often require a crew of people to conduct the work, while such requirements would not be necessary with scent-detection dogs. Finally, capture-based techniques or visual surveys can be difficult to implement in remote locations, or in deep, densely vegetated, or highly turbid waters (Lake, 2013ab, Collier & Grainger, 2015). In contrast, water samples for canine assessment could be collected with ease by simply extracting small amounts of water from the shoreline of a waterbody.

While scent detection dogs may offer several advantages as a carp surveillance method, it is important to recognise potential limitations associated with their use. Firstly, dogs, like

eDNA, would not be able to provide demographic data on the target species. Obtaining information on the sex, age, reproductive status, and location, which can be accomplished through capture-based techniques, can help fishery managers decide what control methods to use (i.e., nets or electrofishing, or both), and where the efforts of these control methods should be focussed (Murphy & Willis, 1996). Secondly, dogs are living organisms and as such there are a variety of factors that may affect their performance. For example, their performance could be influenced by their health, lifestyle, exercise regime, diurnal rhythm, diet, age, breed, or sex (Miller & Bender, 2012; Hall *et al.*, 2015; Jenkins *et al.*, 2016; Riemer *et al.*, 2018; Troisi *et al*., 2019; Fattah & Abdel-Hamid, 2020; Kokocińska-Kusiak *et al.*, 2021). Gazit *et al.* (2003) found that strenuous activity resulted in reduced detection abilities for explosive detection dogs, while Myers *et al.* (1988) found that infection by canine parainfluenza virus resulted in dogs having lower olfactory sensitivity. Due to individual variation in the dogs scenting abilities, dogs may indicate differently on the same sample, causing uncertainty in the results. However, a consensus-based approach (i.e., where the final determination of a target's absence/presence is based on the collective response of the majority of dogs) has been used in other scent detection studies (Reither *et al.,* 2015), and could be used to resolve this issue. Contamination is a factor that can not only limit the detection success of eDNA, but also the detection success of scent detection dogs (Furton & Myers, 2001; Lazarowski *et al.,* 2020). If samples are not handled or stored correctly, they can become contaminated, increasing the probability of the dogs performing false positive or false negative detections (Furton & Myers, 2001; Lazarowski *et al.,* 2020). It is also possible that the distribution of the fish in the waterbody may influence the dogs' detection performance. If the target organism is present in high densities, its odour is likely to be evenly distributed throughout the water body, improving the chances of detection. Comparatively, if the organism is present at low densities or spatially clumped, then the probability of detecting the target organism's odour may be reduced. Finally, dogs, in contrast

to all existing survey techniques, require both initial and ongoing (maintenance) training, which can take time, skilled labour and resources. Depending on the frequency and intensity of maintenance training, this could substantially increase the cost of using scent detection dogs as a detection method.

All detection methods have their limitations, and this section illustrates that dogs are no exception. However, despite these limitations, the application of laboratory-based scent detection dogs has the potential to mitigate several issues associated with some current invasive fish detection techniques. For example, dogs could provide a non-invasive method that is potentially more time and cost-efficient than existing surveillance techniques (Homan *et al.*, 2001; Smith *et al.*, 2001; Grimm-Seyfarth *et al.*, 2019; Grimm-Seyfarth *et al.*, 2021). If used for surveys, dogs could improve researchers' ability to rapidly and extensively survey waterbodies for new invasions of carp.

5.4 Project Limitations

The use of pet dogs meant that the research project did not incur costs associated with permanently housing the dogs. It also meant that the dogs spent non-working periods at home in an enriched environment. However, like all methodologies there were limitations associated with the pet dog approach. For example, the dogs occasionally missed experimental sessions due to their owner going on vacation or having competing commitments. Depending on the length of the break, this was often accompanied by a temporary decrease in the dogs' detection performance. In addition to missed experimental sessions, two owners withdrew their dogs from the study due to changes in circumstances. Consequently, new dogs had to be recruited and trained, which took considerable time and resources. Similar limitations were also reported by Rutter *et al.* (2021) who explored a volunteer-based model of conservation detection dog training and deployment.

The automated apparatus negated issues associated with handler cueing and delayed reinforcement, but as a prototype, occasional faults occurred such as failing to record the dog's indication response, failing to provide reinforcement, jamming between samples, skipping segments, and segment flaps remaining open after a sample assessment. In most instances, these faults had a negligible impact on the dogs' detection performance and could be fixed by the researcher. However, in rare instances, a technician was required as the faults interfered with data collection and/or elicited frustration behaviours in the dogs (e.g., the dogs would refuse to work). Depending on the severity of the problem, data collection was halted for hours or even days.

Traditional methods of scent detection, such as scent line-ups, allow the samples odours to disperse freely around the experimental room, increasing the chance of cross contamination. To minimise this issue, the samples in this study were held in an enclosed chamber. However, research has revealed that these chambers may not be completely airtight. Edwards (2019) placed 99.5% ethyl acetate in one of the apparatus segments and then sampled air from an adjacent segment. A small amount of ethyl acetate (0.03% of the original concentration) was found in the adjacent segment. Given that dogs can detect odourants at extremely low concentrations (Krestel *et al.*, 1984; Walker *et al.*, 2006) it is possible that this leakage may have caused some of the false positive indications observed in this study.

Due to practical constraints, the number of dogs that could be trained at any given time was limited to five. Ideally, five dogs would have participated in each research chapter. However, this was not possible, as some dogs were withdrawn by their owner due to changes of circumstances or by the researcher due to unsuitability, while others finished their training at different times. As a result, only one dog participated in Experiment One (Chapter 2), four dogs in Experiment Two (Chapter 3) and three dogs in Experiment Three (Chapter 4).

Ten of the 15 dogs that passed the initial selection criteria did not progress past the training phase. This high rate of failure could have been a result of the initial selection criteria not being strict enough (Lazarowski *et al.,* 2023). During the initial interview the dogs were assessed on their arousal levels, sociability with other dogs, and their motivation toward food. However, most dogs were withdrawn from the study due to their inability to work independently with the apparatus. This suggests that greater emphasis should have been placed on the dogs' level of independence during the initial suitability assessment. Having a more refined selection criteria could have reduced the number of dogs withdrawn and improved the project's efficiency (Lazarowski *et al.,* 2023).

It has been demonstrated that dogs trained using odour mixtures generally perform better in the detection task than dogs trained on pure odours (Hall & Wynne, 2018; Kokocińska-Kusiak *et al*., 2021). In Chapter 2 and Chapter 3 the dogs were exposed to water samples that contained either carp, goldfish or no fish scent. In hindsight, it may have been beneficial to include mixed carp and goldfish samples into the training regime. Exposing the dogs to these mixed samples could have improved their ability to spontaneously recognise the target scent when they were exposed to lake water which contained an array of background odourants (i.e., the scent of other fish species etc.) and varied in water quality and carp biomass.

Finally, all fish used in the experimental testing were taken from a large aquarium that contained a minimum of 10 carp and 10 goldfish. Since new fish could not be collected each time a thorough tank cleaning was performed (i.e., once a month), fish that had already been used in experimental testing were placed back into the large aquarium. Although fish selection was random, it is possible that subjects previously utilised in experimental testing were re-selected, and thus the dogs' may have been exposed to a particular fish's scent more than once throughout the duration of this thesis. Re-exposure to an individual's scent can be problematic in scent detection research, as dogs have impeccable olfactory memory and can learn the scent

of a specific individual or sample, rather than the general odour of that species or target (Elliker *et al*., 2014; Johnen *et al*., 2014). Despite visually analysing Experiment One's (i.e., Chapter 2) daily session data to evaluate the impact of fish change on the dog's detection performance, and discovering no discernible change in performance after fish replacement, odour memorisation cannot be ruled out as a potential confounding factor as: (1) the sample size from this experiment was small (i.e., one dog, and only four fish changes were performed throughout the experimental testing period), and (2) the researcher did not know if fish associated with each change had been previously utilised in experimental testing. To negate the issue of odour memorisation, it is recommended that new fish be placed into experimental tanks each time a thorough tank clean is performed in future research.

5.5 Future Recommendations

The results of this thesis have demonstrated that dogs can detect carp scent in water samples collected from laboratory aquaria or natural lakes. While these results are promising and indicate that dogs could one day be used to detect incursions of carp or other invasive aquatic species, further research is required before they can be deployed as a surveillance method.

The threshold concentration for carp scent in natural waters should be determined, as water sourced from freshwater ecosystems likely contains more background odours than aquaria water. These background odours could potentially mask or overpower weaker concentrations of carp scent, thereby impacting the dogs' detection threshold and affecting dogs' potential use as an early detection tool. This effect has been alluded to in previous studies. For example, Hall *et al*. (2013) examined the impact of reinforcer location on a dog's ability to acquire a target scent. They discovered that dogs trained with trials where the experimenter supplied the reinforcer (food) after a correct response performed significantly better than those trained with trials where the reinforcer (food) was buried under the target scent. Whilst the exact reason for this decreased detection performance in food-buried trials was unknown, the

authors suggested that it was likely a result of the food odour overpowering the scent of the target odour, reducing its detectability.

Dogs' scent detection behaviour can undergo extinction if the task becomes too difficult, or they do not receive reinforcement for extended periods (e.g., Porritt *et al.*, 2015; Crawford *et al.*, 2022). For example, if water samples were sent to a scent-detection laboratory, the status of the samples (i.e., target scent present or target scent absent) would be unknown and consequently reinforcement for these samples would not be possible. To avoid the extinction of detection behaviour during operational testing, the sample array would need to contain a known quantity of reinforced baseline target samples (Lazarowski *et al.*, 2020). However, the optimum ratio of such samples needs to be determined. Too few could still result in extinction of the desired behaviours as seen in Crawford *et al.* (2022), while too many could result in loss of processing efficiency.

Analysis of the chemical olfactory profile of the water samples could allow the researcher to determine what volatiles the dogs may be using to identify the target scent. Once this has been established the researcher could evaluate when these odours reach headspace equilibrium in their respective segment. Determining this could improve dogs' ability to detect the target and thus their overall reliability as a detection method. Chemical analysis could also provide insight into the reasons behind changes in the dogs' detection performance, such as a decrease in performance on a water sample from a specific lake. In such cases, the researcher could evaluate and compare the chemical profiles of all lake samples to determine potential volatile compounds responsible for the decrease in detection performance.

Once the methodology has been finalised and the dogs are at a stage where they can be used as a surveillance method for carp, a cost analysis should be performed to determine if laboratory-based canine scent detection is a cost-effective approach to invasive fish detection. Potential factors that should be taken into consideration when performing this analysis are the

time, labour and equipment costs not only required to perform operational testing, but also to train the dogs. If dogs are removed from the programme due to illness, unsuitability, or owner withdrawal, the time and resources lost from this would also need to be factored into the analysis. Finally, pet dogs are almost certainly more cost effective than dogs permanently housed at the scent detection facility; however, there are still costs are associated with their use. For example, owners would require remuneration for bringing their dog into the laboratory each week.

5.6 Conclusion

The research described in this thesis has laid the groundwork for the potential use of dogs as invasive carp detectors by demonstrating that dogs can detect carp in aquaria and naturally sourced water with similar sensitivity levels as eDNA. It is hoped that these results and future recommendations will help scientists to develop a cost effective, efficient, and reliable method of invasive fish detection. The development of such a method could enhance researchers' or other interested parties' ability to regularly or extensively monitor freshwater ecosystems for carp or other invasive aquatic organisms, ultimately improving their ability to preserve freshwater ecosystems for future generations.

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Appendices

Appendix A: Participants

Ruby
Age: 7 years
Breed: Labrador Retriever x Border Collie
Participated in Chapters: 2, 3, 4

Harlee
Age: 1 year
Breed: Labrador Retriever
Participated in Chapters: 3 & 4

Marley
Age: 5 years
Breed: Siberian Husky X
Participated in Chapters: 3

Sabi
Age: 7 years
Breed: Labrador Retriever
Participated in Chapters: 3 & 4

Aspen
Age: 2 years
Breed: Labrador Retriever
In-training

Luna
Age: 5 years
Breed: Border Collie X
Withdrawn

Louie
Age: 1 year
Breed: Golden Retriever X
Withdrawn

Bronte
Age: 4 years
Breed: Australian Kelpie X Staffordshire Bull Terrier
Withdrawn

Ella
Age: 10 months
Breed: Border Collie
Withdrawn

Lexi
Age: 1 year
Breed: Springer Spaniel X Cocker Spaniel
Withdrawn

Lotti
Age: 4 years
Breed: Cattle Dog X
Withdrawn

Mica
Age: 7 years
Breed: Cattle Dog X Heading Dog
Withdrawn

Scout & Holly
Age: 5 (Scout) & 3 (Holly)
Breed: Border Collie X (both dogs)
Withdrawn

Tripp
Age: 11 months
Breed: Heading Dog X Border Collie
Withdrawn

Note: Ollie an 11-month-old French Brittany also participated in this study, but no photos were taken by the researcher

Appendix B: Co-authorship Form

THE UNIVERSITY OF WAIKATO
Te Whare Wānanga o Waikato

Co-Authorship Form

Postgraduate Studies Office
Student and Academic Services Division
Wahanga Ratonga Matauranga Akonga
The University of Waikato
Private Bag 3105
Hamilton 3240, New Zealand
Phone +64 7 838 4439
Website http://www.waikato.ac.nz/sasd/postgrad

This form is to accompany the submission of any PhD that contains research reported in published or unpublished co-authored work. **Please include one copy of this form for each co-authored work.** Completed forms should be included in your appendices for all the copies of your thesis submitted for examination and library deposit (including digital deposit).

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter Two

Collins, M. A., Browne, C. M., Edwards, T. L., Ling, N., Tempero, G. W., Gleeson, D. M., Crockett, K., & Quaife, J. (2022). How low can they go: A comparison between dog (*Canis familiaris*) and environmental DNA detection of invasive koi carp (*Cyprinus rubrofuscus*). *Applied Animal Behaviour Science*, 255, 105729. https://doi.org/10.1016/j.applanim.2022.105729.

Nature of contribution by PhD candidate	Designed study with input from supervisors; conducted scent detection dog experiment and developed appropriate methods; analysed scent detection dog data and wrote draft manuscripts for input from co-authors.
Extent of contribution by PhD candidate (%)	82%

CO-AUTHORS

Name	Nature of Contribution
Clare Browne	Provided science advice and commented on draft manuscript.
Timothy Edwards	Provided science advice and commented on draft manuscript.
Nicholas Ling	Provided science advice and commented on draft manuscript. Helped with the analysis of eDNA data.
Grant Tempero	Provided science advice and commented on draft manuscript.
Dianne Gleeson	Provided science advice and commented on draft manuscript.
Kimberly Crockett	Conducted the eDNA experiment.
Jesse Quaife	Commented on draft manuscript.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

Name	Signature	Date
Clare Browne		10/8/23
Timothy Edwards		10/8/23
Nicholas Ling		27/09/23
Grant Tempero		12/6/23
Dianne Gleeson		17/08/23
Kimberly Crockett		21/08/2023

July 2015

, __esse_Qua_1re_ @.4 108/08/23

Appendix C: Standard Operating Procedures

1. Recruitment Information & Induction Interview

Recruitment Checklist:

Interested owner's email dogs@waikato.ac.nz

email redirected to person in charge of recruitment who will follow up with preliminary email

When Consent forms returned and signed, recruiter will invite suitable dogs/owners for interview

Remind owner of interview rules: no children allowed, can bring another adult who may also be involved in picking/dropping off dogs, please bring collar, lead (dog gear).

During interview: review Initial Enquiry Form and observe behaviour (see checklist)

weekly timetable indicating owner's availability, advise the owner that you will keep them updated on dog's progress/behaviour at the lab. Advise what would happen in an incident/fire event, sign-in and out process, and ask the owner to keep you informed of their dog's health and wellbeing (food restrictions, if necessary), and that dogs can be removed from the project at any point in time.

after interview: follow up email with results and reminder of participation schedule if they will participate.

Recruitment

- Initial contact with the Scent Detection Research Group will be established via the official dog lab email address: dogs@waikato.ac.nz controlled by the laboratory manager.
- The laboratory manager will forward the message to the researcher who is currently responsible for recruiting dogs.
- A preliminary email from the researcher will be sent to the prospective owner, including the following three forms: Initial Enquiry Form, Participant Information Form, and Consent Form.
- When these forms have been filled out and returned, the person running that project will reply to the owner via email with the offer of an initial meeting at the laboratory.

- Children are not allowed at the laboratory. However, owners are welcome to bring any other interested adult who might end up picking up or dropping off the dog.
- During the preliminary meeting, start by reviewing the Initial Enquiry Form that the owner submitted and discussing any points that may need to be discussed (e.g., mild anxiety or shyness around other dogs). Then, follow the dog interview checklist (below) to examine the suitability of the dog for the laboratory research and environment.
- After 30 minutes there should be consensus between the owners and the researcher about whether or not to proceed with training.
- If the dog is a good fit, confirm the days/times that the dog is available and make plans for the dog's first visit (or let the owner know that we will be in touch to make these arrangements if the dog will work with another researcher, and provide the relevant information).
- Let the owner know how/where they should enter the building (depending on the project). Explain how they should get your attention (text, knock, or doorbell). Provide any other information that they need to know.

Example of the Initial Enquiry Form:

DOG BEHAVIOUR RESEARCH
Initial Enquiry Form

THE UNIVERSITY OF WAIKATO
Te Whare Wānanga o Waikato

Thank you for your interest in our dog behaviour research. We are looking for dogs who enjoy going to new places and meeting new people – and who really like working for food. We have some other criteria for potential research participants, so if you are interested in your dog possibly taking part, please provide the following information.

Is your dog fully vaccinated (standard vaccines: distempter, hepatitis, parvovirus)? **Yes / No**

If **no**, please explain briefly: _____

_____

_____

Does your dog enjoy meeting new people? **Yes / No**
E.g., are they friendly and comfortable around strangers?

If **no**, please explain briefly: _____

_____

_____

Is your dog comfortable being handled by other people? **Yes / No**
E.g., is your dog happy to be touched on their body, neck, head, tail, paws, etc.?

If **no**, please explain briefly: _____

_____

_____

Is your dog comfortable going to new places? **Yes / No**
E.g., is your dog relaxed and happy (showing no signs of stress) when you go somewhere new?

If **no**, please explain briefly: _____

_____

_____

Is your dog comfortable when you leave them, including at home alone and new places? **Yes / No**
E.g., is your dog relaxed and happy (showing no signs of stress) when you leave them?
(Dogs may be left alone at our training facilities but never for more than 30 minutes at a time so we would like to know if they might have any separation-type anxieties.)

If **no**, please explain briefly: _____

_____

_____

Does your dog like working for food? **Yes / No**

If **no**, please explain briefly: _____

_____

_____

Can your dog eat any food, including kibble (biscuits) and different kinds of meat products? **Yes / No**

If **no**, please explain briefly: _____

_____

_____

Is your dog comfortable with people getting near their food? **Yes / No**

E.g., if your dog has shown any aggression (freezing, growling, snarling, biting) around food, please select 'no'.

If **no**, please explain briefly: _____

_____

_____

Is your dog friendly towards other dogs? **Yes / No**

E.g., if your dog has shown any aggression or fear towards other dogs, please select 'no'. (We will not necessarily have more than one dog at the training facility at once. If we do, it will be with permission of all owners and the dogs will be contained separately.)

If **no**, please explain briefly: _____

_____

_____

Is your dog comfortable with unexpected/loud noises, such as beeping sounds? **Yes / No**

If **no**, please explain briefly: _____

_____

_____

Is your dog free of medical conditions that could be aggravated by repetitive walking? **Yes / No**

E.g., if your dog has any joint or other problems that might be affected, please select 'no'.

If **no**, please explain briefly: _____

_____

_____

Would you be able to drop off and pick up your dog in the morning/afternoon so that your dog spent just half a day with us (our facility is at the University of Waikato main campus)? **Yes / No**

Please indicate which times are more convenient: _____

_____

We want to make sure that all dogs enjoy participating in our research. If you answered "no" to any of these questions, this may indicate that your dog is not suitable for some of this research; however, it does not necessarily exclude them from taking part. A researcher will be in touch with you to discuss the information you have provided here. Thank you for taking the time to complete this form.

Please email this form to:

Interview Checklist

	Step	Notes
	Welcome the owner and the dog into the kennel room; keep the dog on the leash.	
	Review vaccine info (take photos). Review dog gear. Review the Initial Enquiry Form that was emailed; only proceed with behavioural tests if there are no disqualifying responses.	
	Confirm that it is alright for you to interact with the dog and see how it responds to your touching/petting, watching for signs of avoidance or anxiety.	
	Remove the leash and let the dog explore the kennel room (ensure all doors are closed), watching for exploratory behaviour, attachment to owner, and anxiety.	
	Confirm that it is alright for you to offer the dog kibble, then offer kibble and observe: does the dog readily eat the kibble? Does it show increased interest in you?	
	Gesture for/ask dog to enter a suitable kennel (check to see if owner has a command), use kibble to lure if necessary; if dog appears comfortable, close the kennel and observe for any signs of anxiety or attempts to escape.	
	Introduce the dog to the kibble dispenser, activate the dispenser and observe, is the dog frightened? Does it readily eat from the dispenser? Activate the dispenser several times and observe to see if the dog learns to approach as soon as it hears the feeder.	
	Make noises with a segment and/or activation of the apparatus in the experimental room. Watch for fear responses.	
	While occasionally activating the kibble dispenser, stand outside of the room and close the door partially. Does the dog remain engaged with the feeder? Does it try to exit the room?	
	Stay with the dog in the kennel room and ask the owner to step outside. Observe for signs of separation anxiety.	
	Put the leash back on the dog; ask the owner to demonstrate this for you if it is a harness or otherwise unusual equipment (try it yourself to make sure you know how to do it).	

2. Automated Scent-detection Apparatus Training

Apparatus setup

Position the apparatus in a room without other objects that might distract the dog. Only the front panel of the apparatus should be accessible to dogs. Moveable partitions may be used to block access to the other sides of the apparatus. The room must have a door that closes/latches and should be equipped with one or more cameras that can be used to monitor the dogs. The computer(s) used to control the apparatus and monitor the dogs should be positioned in an adjacent room.

Training

Early training sessions should not exceed 10 minutes. At the first sign of fatigue or disinterest terminate the session. Ideally, a session should be terminated after a correct response and reinforcement.

1. ***Establishment of Conditioned Reinforcer***

Each time the dog approaches the feeder use the wireless remote to manually dispense food. Once the dog has approached the feeder 3 times in a row, within 3 s of the released reinforcer, progress to the next stage of training.

2. ***Shaping: Port Entry***

Manually reinforce each successive approximation that is closer to the desired behaviour (i.e., the dog placing its nose in port; process referred to as shaping; refer to port entry in the shaping guidelines). When necessary, use prompts (e.g., hand signals) to help aid progression. All prompts must be phased out before continuing to the next training phase.

Once the dog has placed its nose into the port take the dog out of the room. Load apparatus with target samples (undiluted carp water). Create a configuration file for the dog. In the configuration file set the indication time 1000 ms and the minimum observation time to 500 ms. Shape the dog to place its nose in the port and hold it there for 1000 ms to activate the feeder. Once the dog has completed a session without prompts, gradually increase the indication time until 1500 ms is reached. At this stage, progress to lever press training.

Note: increments in indication time may vary depending on the dog. In most instances progress in 250 ms intervals.

3. ***Shaping: Lever activation***

Turn apparatus off. Use the method of differential reinforcement of successive approximations to shape lever pressing (refer to lever press in the shaping guidelines section for shaping procedure). The topography of the lever press may be dog specific (i.e., chest press, paw press, nose press). Once the lever has been activated 10 times without prompts, progress to discrimination training.

Note: Deliver all reinforcements manually via wireless remote control.

4. ***Discrimination Training***

Place target (undiluted carp water) and non-target (no fish scented water) samples on the apparatus in an alternating fashion. Always put a target sample first. Enter the status of the samples into the configuration file (1 or 9 = target sample; 2 or 3 = non-target scents) and set an indication threshold of 1500 ms and an observation time of 500 ms. Put the dog in the experimental room and stand adjacent to the apparatus (i.e., by the lever). If after 20 s the dog

has displayed no interest in the apparatus, prompt it toward the port. Apply the same rule for the first negative sample, albeit prompt toward the lever if no interest is displayed. Gradually fade prompts out until the dog is working the apparatus autonomously. Once the dog has completed one session without verbal or physical reminders, randomise the sample arrangement. When the dog has achieved a sessional hit rate on target samples and correct rejection rate on non-target samples of ≥80%, gradually increase the indication time until the target threshold is reached (typically 4 s but can vary per dog) and begin to remove yourself from room. At this point, additional rounds may be added (i.e., the samples can be presented two or more times in one session) or the sample concentration, type, or distribution (e.g., positive sample prevalence) can be changed systematically as required.

Note: All samples should contain a high concentration of the target sample to ensure for easy discrimination. A maximum of six sessions should be performed on each sample arrangement. Once the dog has completed six session re-randomise the samples.

Trouble shooting:

Problem	Solution
Dog has poor performance	• Ensure the dog is not experiencing any health-related issues. Always deal with these first. • Evaluate effectiveness of reinforcer (i.e., kibble). This could be done with a simple preference test or attempting to shape a simple response by the dog. If palatability is not the issue, confirm that the owner is not over feeding the dog (i.e., the owner should be feeding half the dog's daily intake). • If the dog regresses and is no longer completing the desired task reliably, re-shape the task (e.g., if the dog continuously fails to press the lever after a negative sample, conduct another lever press shaping session in isolation). • Check factors related to sample quality (i.e., make sure samples have been prepared according to the SOP and cross contamination did not occur). • In a case where the dog's performance does not improve, consult supervisor and/or cease participation in the study.

Shaping Guidelines

Introduction

This document outlines the basic training for training by successive approximations. Normally each step would be completed 3 times consecutively, before progression to the next stage of training. Complete more trials per stage if the dog is not showing reliable performance. Always end with a positive reinforcement and keep trials between 5-10 minutes.

Procedure

The researcher should position themselves adjacent to the lever and avoid eye contact with the dog to reduce the potential of unintentional cueing. Prompts (physical or verbal) can be used to facilitate training, but all prompts should be phased out before the training of the desired behaviour is complete.

Port entry

1. Turn apparatus on. Open configuration file on the computer controlling the apparatus and set the indication time to a high number (i.e., 1000000 ms). Once 'start' is pressed on the computer program this will allow reinforcement to be given manually but also allow a 'beep' sound to be produced by the apparatus.
2. Reinforce moving further and further away from the feeder until the dog is reliably approaching the apparatus (other side of the room)
3. Reinforce attending to the apparatus (putting nose near any part of the front panel).
4. Reinforce placing nose near port.
5. Reinforce placing nose in port.
6. Reinforce breaking the beam for any length of time (indicated by "beeping" sound).
7. Reinforce touching the segment flap with nose.
8. Reinforce pushing the flap inwards.

Shaping of lever press

1. Turn off the apparatus.
2. Reinforce any movement toward lever.
3. Reinforce movement of nose or paw toward lever (as appropriate).
4. Reinforce any contact with the lever (nose or paw, as appropriate).
5. Reinforce any movement of the lever.
6. Reinforce movement of the lever that produces the "click" (microswitch closure).

3. Acid Washing

This SOP can be followed for equipment washed in both nitric acid (HNO_3) or hydrochloric acid (HCl).

Preparation

1. Put on covered shoes, safety glasses, head shield, laboratory coat, acid-proof apron, gloves (blue nitrile gloves underneath nitron gloves; always check gloves for holes before use).
2. Ensure equipment needed for acid washing (i.e., tongs, plastic tray for placement of the pre-acid washed equipment) and the clean-up ((paper towels, neutralising agent (sodium bicarbonate), sealable plastic bag)) are in or adjacent to the fume hood containing the acid, respectively.

Placing in Acid Bath

1. Use metal tongs to pick up and place the equipment into the acid. Make sure the equipment is fully submerged with no air bubbles present.
2. Once all equipment is placed in the acid, place bicarbonate on any residue acid, and then wipe away with a paper towel. Put used paper towels into a zip-lock bag and dispose of it appropriately.
3. Thoroughly rinse the nitron gloves and metal tongs under running water for 3-5 minutes to remove any acid.
4. Leave equipment in acid bath for minimum of 8 hours.

Taking out of Acid Bath

1. Put on PPE, and prepare equipment, as outlined in the preparation section.
2. Three quarter full a 20 L bucket with reverse osmosis (RO) water. In the case of accidental overflow, ensure that the water bucket sits within a plastic tray.
3. Carefully remove equipment from the acid using the metal tongs. Empty equipment of acid by pouring it back into the bath.
4. Place the acid-soaked equipment into the bucket of water. Ensure all equipment is fully submerged.
5. Once all equipment is removed from the acid bath, let it sit in the RO water for 10 minutes.
6. When 10 minutes has exceeded, plug the sink. One by one rinse the equipment three times with RO water.
7. After thorough rinsing place the equipment in the oven to dry.
8. Place one cup of sodium bicarbonate in the sink containing the water used to rinse the equipment. Test water pH with litmus paper. Continue to place neutralising agent into water and test with litmus paper. Once a pH of 7 is reached, release plug for water to drain.
9. Rinse gloves and tongs under RO water and place them back in their appropriate positions.

Taking out of Oven

1. Wear clean latex gloves underneath oven mitt.

2. Place equipment into un-used plastic bags, or containers allocated to a specific sample type.
3. Take equipment back down to the Scent Detection Research Facility.

Neutralising used acid

1. Put on PPE as outlined in the preparation section.
2. Place sodium bicarbonate, litmus paper, 20 L bucket, glass beaker, and stirring rod (glass) in the fume hood containing the acid.
3. Using the glass beaker to transfer a small amount of acid into the 20 L bucket.
4. Progressively place small amounts of sodium bicarbonate into the bucket containing the acid. Mix with stirring rod with each addition.

Note: Do NOT place large amounts of sodium bicarbonate in the bucket as on contact with acid, bicarbonate reacts and creates bubbles that can expand and thus overflow out of the bucket.

5. Test the solution in the bucket with litmus paper. When pH of approx. 7 is reached, the acid is neutralised.
6. Once neutralised, pour solution down sink.

Formulating Acid Solution

Nitric acid

1. Put on PPE as outlined in preparation section.
2. Place glass bath allocated to hold the acid in a plastic tray in the fume hood.
3. Carefully pour acid into glass bath.
4. Place lid on acid bath.
5. Naturalise any spilt acid with sodium bicarbonate and wipe residue clean with paper towel. Place used paper towels in a sealable plastic bag and dispose of it appropriately.

Hydrochloric acid (10% v/v)

1. Put on PPE outlined in preparation section.
2. Fill buckets allocated to hold the acid with Ultrapure water. The amount of water is dependent on the quantity of solution required and the bucket size. For example, if the researcher wanted to fill a 20 L bucket with 15 L of 10% v/v hydrochloric acid, 13.5 L of ultrapure water would be required.
3. Place the water filled bucket(s) allocated to hold the acid into fume hood, alongside a glass beaker, and stirring rod (glass).
4. Measure the required amount of acid using the glass beaker. Based on the above example, 1.5 L of acid would be required.
5. Gradually place acid in the bucket containing the water. Stir when placing acid in the bucket.
6. Once formulated leave acid baths in fume hood or carefully remove and place in an appropriate space within the laboratory.
7. Ensure all buckets are labelled appropriately.

IMPORTANT REMINDERS:	
Hydrochloric Acid	Nitric Acid
• Allocate a specific bath to each sample type (i.e., on bath for koi, another for goldfish). • Allocate specific equipment to each sample type and thus acid bath (i.e., gloves, tongs, soaking buckets etc.). • Allocate specific shelves in oven to a sample type. • Replace acid and acid bath (i.e., bucket) every 4-6 months	• Only place glassware in the nitric acid. • NEVER place plasticware in the nitric acid. • Replace acid with a new solution every 6-months.

4. Sample Collection, Tank Cleaning & Bench Preparation procedure

Purpose

This SOP provides guidelines and standardised procedures to be adopted during collection of water samples and cleaning of aquaria at the University of Waikato's Aquatic Research Centre. Only individuals who have been inducted into the facility by authorised personal can carry out these procedures.

Sample Collection

1 Twenty-four hours prior to sample collection the tank must be drained (water level must remain above fish), scrubbed clean with a scouring pad, and all tanks containing fish must be refilled to a water level that equates to 15.5 g of fish/litre. Once the fish tanks are at this level turn water flow into the tank off. Leave the control tank on low flow. *Note: To determine the water level the fish must be weighed prior to being put into the tank. Using the water depth calculator (found in pest fish file in the scent drive), enter the fish's weight in the 'biomass (g)' column. The water in litres and the depth will be calculated automatically. Using a measuring tape and a permanent marker, vertically measure the number of centimetres calculated in the 'Depth (cm)' column on the fish tank. Mark this measurement on the tank with the permanent marker and then fill the tank to this level.*

2 After 24-hours, commence in sample collection. For this, use a clean nitric acid washed beaker. Collect water with the beaker and place it into the collection bottle allocated to that sample type. Screw the lid on tight to prevent odour leakage. Place the full collection bottle into a clean plastic bag and used beaker into a plastic bag allocated to used beakers. These will be taken to the Scent Detection Research Facility for sample preparation and the R block laboratory for nitric acid washing, respectively.

3 If the fish tanks are to be used for sample collection the following day, allow water to enter the tank, fill the tank and then repeat the instructions outlined in section 1.

4 If the tanks are not being used for sample collection the following day, leave the tank on low flow, and feed the fish.

5 Clean all fish tanks the day after feeding following the same procedure as outline in section 1, albeit leave the tanks on low flow after cleaning.

Bench Preparation

6 Fill and boil the jug. Pour boiling water over entire bench. Repeat. Spray bench with Isopropyl alcohol solution (concentration = 50% Isopropyl:50% water). Wipe benches dry with paper towels.

7 Put paper towels down on the bench.

8 Place sample containers in allocated positions on the bench; no fish on the right side of the bench, goldfish in the middle, and koi samples on the left-hand side of the bench.

9 Put an adhesive strip in an allocated position on each group of containers (e.g., no-fish = horizontal, near containers edge; goldfish = vertical near containers edge; koi = middle near containers edge).

5. Preservation Experiment Sample Preparation

This standard operating procedure (SOP) provides guidelines and standardised procedures to be adopted when performing the preservation experiment (Chapter 3).

General Rules

- All water samples used for the preservation experiment must be collected, prepared and the selected preservation method applied seven or eight days before assessment by the dogs.
- Samples must be collected or prepared in the order of negative to positive (e.g., no-fish scent (negative, non-target), goldfish scent (negative, non-target), koi carp scent (positive, target))
- Gloves must always be worn and changed between handling of different sample types or equipment.
- Equipment must be allocated to each sample type to avoid cross contamination.

Sample Preparation

Baseline samples

1 Measure and pour 100 mL of no-fish scent water into all containers set aside for use in the experiment.
2 Place an additional 100 mL of no-fish scent water into a spare sample container (clean).
3 Using a pipette, extract 200 µL of no-fish scent water from the spare container and place it into each of the containers allocated to the no-fish scent sample type.
4 Place the no-fish scent samples on the scent detection apparatus in a pre-arranged randomised order, with segments.
5 Repeat steps 1 to 4, however, instead of placing 100 mL of no-fish scent water into a spare plastic container and extracting 200 µL using a pipette, place 100 mL of goldfish and then koi carp water into a spare plastic container, respectively. Change pipette tips between sample types.

Preserved samples

1 Prepare the ‘preserved’ samples seven and eight days before they are assessed by the dogs.
2 Allocate a plastic container to each sample type (e.g., no-fish, goldfish, koi). Place the plastic containers in an assigned position on the bench (e.g., no-fish = left-hand side, goldfish = middle, koi = right-hand side of bench).
3 Place 100 mL of no-fish scent water into the plastic container allocated to this sample type.
4 Using a pipette, extract 2 mL of the no-fish scent water and place it into the Nalgene bottle containing 1 L of no-fish scent water.

Note: This extraction quantity was selected as it is the concentration equivalent to that used for the baseline samples (i.e., baseline samples = 200 µL in 100 mL water; Preserved samples = 2 mL in 1000 mL, so 200 µL per 100 mL).

5 Invert the sample three times and then subject the sample to the appropriate preservation method.

Note: If you are using adding the chemical preservative invert the sample three times after its addition. Refer to chemical preservative preparation section for more detail on how to make the chemical solutions, and the quantity of chemical required per 1 L of water.

When freezing or chilling the samples ensure that they samples are keep in separate positions within the freezer or fridge, respectively, to avoid cross-contamination.

6 Repeat steps 3 to 5, however, place 100 mL of goldfish or koi water, respectively, into a plastic container, extract 2 mL and place it into 1 L of no-fish scent water.

7 On the day of presentation to the dogs, add 100 mL of each sample type into the containers set aside for the experiment. Place the samples on the scent detection apparatus in a pre-arranged randomised order with a segment.

Note: Always prepare and place the samples on the SDA systematically (i.e., prepare and place no-fish scent samples on the SDA first, followed by goldfish and the koi carp samples).

Remove samples from the freezer 24 hours prior to presentation to the dogs.

Chemical Preparation

Chemical reagents are frequently added to water samples when storage at low temperatures is not possible. In this study we used an antimicrobial agent, Potassium sorbate, as it is commonly used to preserve food stuffs, its non-toxic to dogs and odourless to humans.

Stock Solution

1 Put a small plastic tray on the scales. Zero the scales and place the pre-specified amount of chemical (i.e., 25 g Potassium sorbate) on the tray.
2 Place the chemical into a volumetric flask and add 100 mL of ultra-pure water.
3 Put cork on volumetric flask.
4 Invert or stir the sample until all solid material has dissolved.
5 Pour the chemical into glass bottle (100 mL)
6 Make a label for each chemical. Ensure the label has the appropriate safety signage.

Quantity of Chemical

This experiment is performed to determine the quantity of chemical required to make the water sample a certain pH.

1 Calibrate the pH probe.
2 Calibrate pH probe with pH 4 buffer if using an acid; pH 7 if using an antimicrobial agent; pH 10 if using a base.
3 Rinse the pH probe with Ultrapure water.

4 Place pH probe into the bottle containing 1 L no-fish scent water and wait for the pH probe to stabilise.

5 Once stabilised systematically add the antimicrobial agent (~5 mL) until the no-fish scent reaches a pH of 7.
Record the amount of antimicrobial agent required to make the water sample a pH of 7.