Canine Accuracy in the Scent Detection and Discrimination of Invasive Fish

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Abstract

The scent-detection accuracy of dogs (*Canis familiaris*) was assessed as they learned to discriminate between water samples from aquaria containing koi carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) or no fish, at systematically diluted concentrations. The study was conducted using an automated apparatus. Three dogs were trained in a go/no-go task where hits on target (koi carp) samples were reinforced and correct rejections of non-target (goldfish and no-fish) samples had no programmed consequences. Conducting a control test indicated that the dogs' accuracy measures were likely influenced by extraneous variables. Consequently, modifications were made to procedures and the apparatus. A multiple-probe design experiment found the dogs were able to detect koi carp scent at all of the concentrations tested (from 0.098 mL/100 mL to 0.00094 mL/100 mL), equivalent to an effective biomass range of between 72.5 and 0.4 kg/ha. No clear relationship between accuracy measures and the systematic dilution of the sample concentration was observed for any of the dogs. A test of the dogs' accuracy with the samples removed from the apparatus suggested that volatiles evaporating from the sampled aquarium water may have been accumulating in the segments as the dogs continued to respond according to the status of the samples that were previously in each segment. Sensitivity was found to be similar across first trials and second trials for target samples. Specificity was found to increase for non-target samples from first trials to second trials. These results have significant practical implications for the use of scent-detection dogs for koi carp detection and the further development of research methods for their training and assessment.

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당신의 도움에 항상 감사하고 있어요. 올해는 당신 덕분에 가장 즐겁게 공부했어요. 힘든일이 있을때도 내가 웃을 수 있게 해줘서.

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Chapter 1: Introduction

Global increases in trade and travel together with efficient transportation facilitate the movement of species and subsequently the occurrence of species incursions (Everett, 2000; Pimentel et al., 2001). Some of these species incursions cause major economic losses estimated at over $300 billion US dollars annually across the United States, the United Kingdom, South Africa, Australia, India and Brazil (Pimental et al., 2001). To lessen the economic burden of unwanted species incursions, early detection and intervention are imperative for preventing the establishment of extensive populations and increasing the likelihood of eradication (Bogich, Liebhold & Shea, 2008; Kirticos, Phillips & Suckling, 2005; Sargisson, Popay, McLean & Crocker, 2010). Using behavioural conditioning principles, dogs (*Canis familiaris*) can be trained to detect and signal on the scent of a species. This may be more useful than some current approaches used in the detection of specific species incursions and in determining the success of eradication measures.

Dog scent detection

Dogs have been used in various scent detection tasks due to their olfactory acuity, biological behavioural tendencies such as prey and play drives, and trainability (Cablk & Heaton, 2006; Lesniak et al., 2008). The olfactory region of a dog's nose is sensitive to many different kinds of volatile organic compounds (VOCs), some at thresholds in parts per trillion such as *n*-amyl acetate (Craven, Patterson & Settles, 2009; Walker et al., 2006). Dogs have been trained to detect specific VOCs related to a range of targets, such as explosives (Furton & Myers, 2001), lung and cervical cancers (Guerrero-Flores et al., 2017; McCulloch et al., 2006), oestrus in cows (Fischer-Tenhagen, Wetterholm, Tenhagen & Heuwieser, 2011), decay-causing microbes in wood (Kauhanen, Harri, Nevalainen & Nevalainen, 2002), human remains (Alexander, Hodges, Bytheway & Aitkenhead-Peterson, 2015), virally infected bovine cells (Angle et al., 2016) and contaminated water (Van De Werfhorst, Murray, Reynolds, Reynolds & Holden, 2014).

Organisms are continually releasing VOCs into their surroundings, and similar assortments of VOCs are released by organisms of the same species rendering them identifiable via a signature scent (Fink, 2007; Goodwin, Engel, & Weaver, 2010; Yuan et al., 2017). Dogs have been found to detect VOCs from live and dead specimens of a species, as well as from a species' residual scent and by-products, such as scats and shed skin (Browne, Stafford & Fordham, 2015; Cablk & Heaton, 2006; Long, Donovan, Mackay, Zielinski & Buzas, 2007; Russell, Beaven, MacKay, Towns & Clout, 2008; Savidge, Stanford, Reed, Haddock & Adams, 2011). Even VOCs released from aquatic species can be detected by dogs as they evaporate from water into the atmosphere (DeShon, Wong, Farmer & Jensen, 2016; Fink, 2007; Quaife, 2018). Research into the ability of dogs to detect and locate the scent of invasive pest organisms has shown promise in aiding management and eradication practices.

Dog scent detection of invasive species

Less than a decade ago, the Redbay ambrosia beetle (*Xyleborus glabratus*) invaded the United States carrying with it a fungus (*Raffaelea lauricola*) which causes considerable damage to plants of the family Lauraceae, such as avocado trees (Mendel et al., 2018; Simon, Mills & Furton, 2017). Mendel et al. (2018) trained three dogs to detect the VOCs released by the fungi present on avocado wood. Initially, the dogs were reinforced for the searching, retrieval and locating of a toy of preference. The novel odour of a synthetic compound was then paired with the toy, and eventually, the synthetic compound odour was replaced with the target odour. During training sessions, each dog with its handler searched an orchard for infected wood samples. The presence of the target odour was indicated by a trained response (dog sitting next to the tree). The dogs correctly detected targets with an average accuracy rate of 99.4%. Testing of the dogs in operational settings showed the dogs were able to detect the presence of the fungus in avocado trees before any noticeable pathogenic symptoms could be observed. This is particularly important as the early treatment of infected plants with fungicides can prevent the progression of the disease. Mendel et al. (2018) showed that dogs are potentially an asset to the avocado industry, detecting infections of *R. lauricola* early on and therefore helping growers streamline control measures.

Similarly, the red palm weevil (*Rhynchophorus ferrugineus*) is an invasive pest that cannot be easily identified during the initial stages of infestation, when control measures are most likely to be successful (Suma, La Pergola, Longo & Soroker, 2014). With no previous scent detection training, dogs were trained over a period of three months to detect red palm weevil scent. Suma et al. (2014) placed the target scent (red palm weevils or larvae and diseased palm plant) inside a tennis ball and reinforced the dogs' retrieval and finding of the tennis ball. The same target scents were then hidden among palm plants and positive indications, either barking or sitting, were reinforced when correct. With handlers blind to the placement of targets, Suma et al. (2014) tested the detection accuracy of each of the three dogs with contained targets arranged among 12 palm plants. The targets contained low numbers of red palm weevil of specific sexes and ages (five live male or female red palm weevils, five pairs of male and female red palm weevils, five larvae, or 30 g of palm plant containing red weevil larvae). The dogs, led by handlers, were able to detect all tested targets with a mean accuracy rate of 78%, with similar rates of detection across the target types.

Depending on the target, it can be useful to train dogs to detect species at a variety of life stages, and to discriminate between these life stages and associated sources of scent. In Europe, Hoyer-Tomiczek, Sauseng and Hoch (2016) initially reinforced indication of only the live larvae of the Asian Longhorn beetle (*Anoplophora glabripennis*). Once reliable detections were occurring, multiple sources of beetle scent were introduced and detections reinforced. Detection of Asian Longhorn beetle scent emanating from larvae, wood shavings and frass, or infested wood and larvae (controlled setting) and a mixture of wood shavings and frass (field setting) occurred with a high degree of accuracy across a large number dogs. In contrast, Pfiester, Koehler and Pereira (2008) differentially reinforced indication of the detection of live sources (bed bugs and eggs) and non-living sources (bed bug faeces, skins, dead bed bugs) of bed bug (*Cimex lectularius L.*) scent. Under controlled conditions, three trained dogs achieved 100% and 90% accuracy in the detection of live bed bugs and eggs, respectively. False positive indications on bed bug faeces, skins and dead bed bugs occurred at a very low rate (<5%).

Generalisation of a conditioned response to the scent of closely related species of the target can occur. For example, Brooks, Oi, and Koehler (2003) tested dogs trained in the detection of Eastern subterranean termites (*Reticulitermes flavipes*) with four other species of termite. It was found that positive indications for all of the four termite species occurred at similar rates to those of the target species. Distinguishing between closely related species is particularly important when an invasive species inhabits an environment with several similar native species (Fukuzawa & Sasahara, 2019) or when targeting a particularly harmful species. For example, Lin et al. (2011) found that dogs trained to detect red fire ants (*Solenopsis invicta*) could discriminate between this species and three other species of ant.

In recent years, a dog and its handler has been employed by New Zealand's Department of Conservation (DOC) as part of their Conservation Dogs Programme in the detection of the invasive Argentine ant (*Iridomyrmex humilis*). Argentine ants are a cryptic species which has been present in New Zealand for more than two decades (Ward et al., 2014). Initial training involved the handler reinforcing sniffing and then sitting next to a target, and providing positive punishment for false indications. In blind trials, 100 vials, of which 30 contained targets or target scent, were arranged in a grid formation. The dog was able to detect vials of Argentine ants and Argentine ant scent, and discriminate between these, empty vials and three different species of ant. Accuracy rates were above 80% across trials for vials containing Argentine ants and Argentine ant scent. False positive indications occurred on 7% of trials for non-target ant vials and 0% for controls (empty vials). The dog has since been deployed in field situations and has been successful at locating Argentine ants where eradication programs are taking place, such as on Great Mercury Island.

Rodents (of genera *Rattus* and *Mus*) are an ongoing threat to sanctuaries and islands of New Zealand where they have been eradicated (Gsell, Innes, de Monchy & Brunton, 2010; Shapira, Buchanan & Brunton, 2011). If an incursion occurs, preventing the establishment of rodent populations is vitally important, but small numbers of rodents can be difficult to detect (Russell et al., 2008; Shapira et al., 2011). DOC dogs specifically trained to detect rodents can quickly locate individual rats and mice across large areas. For example, one dog was found capable of detecting caged rats at up to 20 metres from the scent source location. It tracked a free-ranging rat within a few hours, that was located several hundred metres from its release point (Shapira et al., 2011). In another experimental study, two dogs trained in the detection of rodents located either a caged mouse or rat in a 32-hectare forest with 86% accuracy across trials. With the rodents removed from the cages, the dogs were able to locate the cages with 77% accuracy across trials (Gsell, et al., 2010).

Dog scent detection can also be applied to invasive plants. Sargisson et al. (2010) used a match-to-sample procedure when training two dogs in the detection of invasive weeds. The dogs were exposed to a sample of the target plant odour and then searched an array of samples consisting of the target plant and several non-targets. Reinforcement was arranged when the dog sat next to the matched sample. In field trials, both dogs could discriminate specific target weeds from the odours of many non-target plants, but detection rates were found to be hampered by unfavourable environmental conditions. For detection of spotted knapweed (*Centaurea stobe*) Goodwin et al. (2010) used discrete trial training. One target sample was placed in a line-up arrangement with up to five other non-target samples. A handler reinforced positive indications when the target sample was located, and incorrect or missed target trials were repeated. As detection accuracy improved, blind and then field trials were conducted. In the field trials, Goodwin et al. compared the performance of three trained dogs with that of humans experienced in the detection of spotted knapweed plants. Human detection teams and each dog and its handler, blind to target locations, searched a grassland area of 3.5 hectares four times over a year. More target plants were located and fewer missed by dogs than from human sampling efforts. The dogs were also found to be more accurate than humans in finding plants of small (67.1% v. 34.7% accuracy, respectively) and medium size (94.4% v. 77.8% accuracy, respectively). Overall accuracy of the three dogs in finding spotted knapweed plants of any size was 81.1% compared to human search accuracy of 58.9%.

A less commonly explored area of research is the utilisation of dogs in the detection of invasive aquatic species. DeShon et al. (2016) tested the ability of four dogs, already trained in the detection of quagga mussels (*Dreissena rostriformis bugensis*), to detect the veligers (larvae) of quagga mussels. One target water sample containing veligers and four non-target water samples were each placed inside a bucket with a perforated lid. The dogs were initially exposed to a high number of veligers in target samples, and a handler arranged reinforcement for correct indications. Once the dogs were correctly detecting the veliger odour, trials in which the concentration of the veligers was systematically reduced began. Samples were arranged in a line and handlers were blind to the target's location. Across four trials with the lowest concentration of 31 veligers in 360 mL of water, all four dogs correctly alerted to target samples and correctly ignored non-target samples. DeShon et al. (2016) concluded that the dogs' detection levels of the veliger odour were sensitive enough to be useful in field detection scenarios.

Research investigating the utility of dogs in the detection of invasive organisms has shown that they can detect organisms present in low numbers, can track scent over long distances, and can be trained to detect a very specific or broad target scent. In New Zealand, additional tools are needed in the detection of the invasive aquatic species, koi carp (*Cyprinus carpio)* (Grainger, 2015a). Thus, scent-detection dogs might be useful in the detection of koi carp when present in low numbers, such as in the early stages of an incursion, assisting in the management and conservation of waterways.

The invasion of koi carp into New Zealand's waterways

The human-mediated invasion of koi carp into New Zealand water bodies likely began during the 1960s (Blackburn et al., 2011; de Winton, Dugdale, & Clayton, 2001; Hicks & Ling, 2015). New Zealand authorities at the time were slow to initiate a response to this invasion (McDowall, 2004) and a koi carp population soon established in the North Island's Whangamarino River (Hicks & Ling, 2015). Koi carp are now legally defined as an unwanted and noxious invasive species of freshwater habitats in New Zealand (Collier & Grainger, 2015; New Zealand Government, 1983; New Zealand Government 1993; Goodman et al., 2014).

A designated koi carp containment area was established in 1990, covering the Waikato and Auckland regions, in an attempt to prevent the expansion of koi carp territory (Grainger, 2015b). Despite these efforts, small, scattered populations can be found throughout the North Island, and an incursion occurred in the South Island in the early 2000s (Collier & Grainger, 2015). Koi carp populations now dominate water bodies of the Waikato region, and with large populations, direct and indirect effects on the environment become more pronounced.

Over the years, the Waikato River has been impacted by the installation of hydroelectric dams and through the agricultural and industrial development of surrounding land (Blair & Hicks, 2012). A growing koi carp population has contributed to the environmental degradation of rivers and lakes in the Waikato Region. In 2005, estimates of koi carp biomass at sites along the Waikato River ranged from 39.6 kg/ha to 307.8 kg/ha. In 2008, koi carp biomass in the Whangamarino River and its surrounding tributaries was estimated to be 325.8 kg/ha (Hicks et al., 2015). Koi carp are benthivorous (foraging on the bottom of a waterbody) and in their search for benthic prey, disturb the sediment bed (Collier & Grainger, 2015; Shormann & Cotner, 1997). Their feeding actions dislodge macrophytes, which reduces sediment stability and suspends sediment and nutrients in the water column (Daniel & Morgan, 2011; de Winton et al., 2001). In an experimental aquarium, large koi carp were found to disturb sediment up to 20 mm deep (Wall, Isley, & La Point, 1996). This behaviour contributes to water turbidity and nutrient enrichment, supporting algal dominance, which can, in turn, reduce light penetration, further harming aquatic plants (Collier & Grainger, 2015; Hicks, Brijs, et al., 2015; Rowe, 2007). Decreasing the koi carp biomass reduces the extent of these effects and may benefit native species. For example, the numbers of native eels (*Anguilla dieffenbachia* and *Anguilla australis*) in Lake Ohinewai had been declining, but following the removal of a large number of koi carp, the numbers of eels increased (Allibone et al., 2010; Daniel & Morgan, 2011; Hicks, Brijs, et al., 2015).

Koi carp are a variant of common carp, of which there are many strains (Xu et al., 2014). Carp is a species capable of establishing populations in a wide range of aquatic habitats, having been introduced to over 100 countries and successfully establishing populations in the majority of these (Garcia-Berthou et al., 2005). Due to genetic and habitat diversity, carp show differences in growth, environmental tolerances and some reproductive characteristics (Oyugi et al., 2011; Tempero, Ling, Hicks, & Osborne, 2006; Xu et al., 2014). However, the environmental impact of invasive carp populations on different aquatic habitats is very similar. For example, studies in the USA and Canada have reported poor water quality associated with carp populations in both experimental and lake habitats (Bajer, Sullivan, & Sorensen, 2009; Fischer, Krogman, & Quist, 2013; Jackson, Quist, Downing, & Larscheid, 2010; Miller & Crowl, 2006; Badiou & Goldsborough, 2010). In Poland, Kloskowski (2011) compared different densities of carp with water quality and found high carp densities were associated with poorer water clarity, reduced numbers of macrophytes, increased turbidity and increases in the numbers of phytoplankton. In Australia, carp are widespread (Koehn, 2004), and research by Driver, Closs, and Koen (2005) found disturbance and suspension of sediment by carp was associated with water conditions of turbidity and high phosphorus and nitrogen concentrations. The environmental disturbances caused by invasive species can potentially lead to permanent ecological change (Norton, 2009); therefore, carp incursions need to be quickly identified before numbers increase and populations become established.

Specific characteristics of koi carp, such as their fertility, growth, longevity and mobility, appear to be advantageous to their continued proliferation. In the lower Waikato region of New Zealand, female koi carp were found to be fecund, producing an average of 299,000 (*SD* = 195,600) oocytes per fish at the start of the spawning season, with some having the potential to spawn more than once, over several months (Tempero et al., 2006). Hybridisation between koi carp and goldfish (*Carassius auratus*), another invasive species in New Zealand, has been found to occur where these species co-exist as they share similar spawning characteristics (Collier, Allan, & Rowe, 2015; Goodman et al., 2014; Pullan & Smith, 1987; Rowe, 2007; Smith & McVeagh, 2005). Genetic variability can potentially enhance survival and adaptation (Hayes et al., 2011). Length-at-age appears to increase, on average, by around 100 mm per year during the first 3 years of life, followed by continued growth at a slowing rate (Tempero et al., 2006). Koi carp mean fork length (measured from the fishes snout to the middle of the caudal fin) ranged from 107.3 mm at age 1 to 700.0 mm at age 12 (Gaygusuz et al., 2006; Tempero et al., 2006). In contrast, fish native to New Zealand tend to be a lot smaller (David, 2015). Age at maturity was 2 and 3 years for male and female koi carp respectively (Tempero et al., 2006). Being highly mobile, koi carp are able to manoeuvre small waterways to gain access to nearby freshwater habitats. When access to an adjacent habitat is restricted, koi carp in the Waikato River have been found to travel distances of over 5 km in search of other habitats (Daniel, Hicks, Ling, & David, 2011). Koi carp were apparent in most accessible water bodies not long after being introduced to the lower Waikato region, and currently inhabit 11,300 ha of the lower Waikato River's water bodies (Byers, 2015; David, 2015; Osborne, Ling, Hicks, & Tempero, 2009).

The invasive potential of a species is also mediated by the receiving habitat (Collier, Leathwick, Ling, & Rowe, 2015). The environment into which koi carp were introduced, the Waikato River, is 450km long, with mild water temperatures (from around 9°C during winter to 23°C in summer months), and many interconnected water bodies providing suitable feeding and spawning habitats (Blair & Hicks, 2012; Collier et al., 2015; Collier & Lill, 2008; Hicks, Brijs, Daniel, Morgan, & Ling, 2015; Tempero et al., 2006). Additionally, there are few piscivorous fish in the Waikato River, limiting the predation of adult koi carp (Osborne et al., 2009).

The dispersal, survival, and reproduction (Blackburn et al. 2011) of koi carp in New Zealand is a major environmental concern. Likewise, the spread of invasive carp populations threatens aquatic environments worldwide (Garcia-Berthou et al., 2005). Koi carp appear to be an adaptable species capable of establishing themselves in many areas of New Zealand. Detection of koi carp at low biomass densities with appropriate follow-up management practices (e.g., application of piscicides such as rotenone to poison fish; Ling, 2002), can reduce, and possibly prevent the negative environmental consequences associated with their presence.

Detection methods

The proliferation of koi carp, particularly in the Waikato region, highlights the threat of further deliberate or unintentional introductions of koi carp to new areas of aquatic habitat in New Zealand. DOC recommends the use of trammel, gill, and fyke nets; visual observation; and electrofishing when sampling for adult koi carp in a water body. Combining all or some of these methods increases the sampling efficiency, especially when sampling for koi carp that are present in low numbers. The choice of sampling method depends on the environmental characteristics of the water body, such as size and depth, and consideration of the consequences to non-target species (Grainger, West, & McCaughan, 2014).

Trammel and gill nets are similar passive sampling methods, entangling fish as they come into contact with the net. These nets are most likely to capture koi carp when set near the surface of a water body in targeted areas (Grainger et al., 2014). To increase detection rates, a number of nets would need to be set up. However, high mortality rates are associated with these netting methods (Lake, 2013), which is of particular concern if native species are also present in the sampling water body. Gill nets also tend to capture fish of a similar size, limiting sampling variety (Hicks, Jones, de Villiers, & Ling, 2015). Fyke nets are another passive sampling method, trapping fish as they enter the net. These nets can be used in aquatic habitats, such as wetlands and streams, and are particularly effective at capturing species that dwell in the benthic zone (Grainger et al., 2014; Hicks, Brijs, & Bell, 2009; Lake, 2013). Again, detection rates can be increased by setting a number of fyke nets in the targeted water body. Unfortunately, the trapping of native eels has been observed to occur frequently with the use of fyke nets and trapped eels can suffer spinal damage (Daniel & Morgan, 2011; Hicks, Jones, et al., 2015).

An additional method that can be used to detect koi carp is visual observation. Using polarising sunglasses, koi carp can be visually detected around the edge of the water body being sampled. The likelihood of visually detecting koi carp increases if specific areas of habitat where koi carp are known to aggregate, such as near macrophyte beds and willow trees, are targeted (Grainger et al., 2014; Hicks et al., 2015). However, visual detection is unlikely to have a high success rate in environments where visual clarity is low, and confirmation of a sighting may be difficult.

Electrofishing involves pulsing electrical current through the water at a voltage strong enough to temporarily stun fish that come into contact with the electric field. Any stunned fish that can be visually observed can then be captured

(Hicks, Brijs, Heaphy, & Bell, 2008; Hicks, Jones, et al., 2015). Electrofishing is an effective method for sampling koi carp of different sizes and can be conducted with a backpack electric fishing machine or an electric fishing boat. This enables the sampling of wadeable and large, deeper bodies of water (Grainger et al., 2014; Hicks et al., 2015). However, there are several limitations associated with its use. Visually locating stunned koi carp can be difficult if the water is murky and some stunned fish may sink instead of float (Hicks et al., 2008; Hicks et al., 2015). Additionally, some koi carp may not be captured if they are beyond, or avoid, the electric field range (Hicks et al., 2015). One of the more concerning limitations of electrofishing is the harm that can be caused to native New Zealand species. Using electrofishing methods, serious effects such as haemorrhaging, spinal damage and mortality have been reported to occur in bycatches of eel, grey mullet and smelt (Hicks et al., 2015).

Instead of relying on the visual identification and capture of target organisms, water samples taken from the targeted water body can be analysed for the presence of species-specific environmental DNA (eDNA) (Furlan, Gleeson, Hardy, & Duncan, 2016; Lodge et al., 2012). Using a species-specific DNA primer and either a polymerase chain reaction (PCR) or quantitative PCR (qPCR) method, detection of a target species can be achieved through the amplification of target DNA (Furlan et al., 2016; Herder et al., 2014).

Many of the limitations associated with traditional sampling methods in the detection of invasive aquatic species can be avoided with the use of eDNA. For example, organisms are not harmed, sampling is not biased to fish of a particular size and visual identification is not required (Bylemans et al., 2017; Hinlo, Furlan, Suitor, & Gleeson, 2017; Lodge et al., 2012). The detection limits of eDNA show its promise in the monitoring of sites and, in particular, for the detection of recent incursions. For example, under controlled conditions, Goldberg, Sepulveda, Ray, Baumgardt and Waits (2013) tested the sensitivity of eDNA analyses with densities of 1, 10, 50, 100 and 200 New Zealand mudsnails in water volumes of 1.5 L. Two days post introduction, eDNA analyses detected the presence of mudsnails at all tested densities. After four days, all mudsnails were removed from each of the 1.5 L water containers. Three days post removal, eDNA analyses were conducted again. Mudsnail eDNA was still detected, including from the sample of water taken from the container that had housed only one mudsnail. Despite the high sensitivity of eDNA analyses, amplification failure of low concentrations (<0.1 target molecules per PCR replicate) of DNA molecules is possible (Furlan et al., 2016). eDNA analyses conducted by Hinlo et al. (2017) failed to detect common carp known to be present in a wetland. Hinlo et al. (2017) suggested the low abundance of common carp and hence the low concentrations of eDNA were likely factors contributing to the false negative result. In addition, the dispersal of eDNA in the environment was found to be irregular. From six creek samples across the wetland, five contained no target (common carp) DNA, but one sample contained a high concentration of target DNA. Likewise, Goldberg et al. (2013) found target eDNA was present in variable concentrations depending on the area of habitat sampled, the distance from live targets and the water velocity.

Once collected, the DNA in environmental DNA samples degrades rapidly, irrespective of how it is kept (Hinlo, Gleeson, Lintermans & Furlan, 2017; Knox, Hicks, Banks, & Hogg, 2008). Analysing samples within 24 hours of collection can reduce false negative results (Hinlo, Gleeson, et al., 2017). Also collecting more, larger samples and performing repeated analyses could reduce false negatives. However, reducing false negative results would also increase costs. eDNA methods require specialised laboratories, equipment and trained personnel to conduct these analyses which have been estimated to cost 35 to 80 USD per sample (Furlan et al., 2016; Goldberg et al., 2013; Hinlo, Gleeson, et al., 2017).

False positive detection errors can also occur when sampling and analysing low concentrations of eDNA (Furlan et al., 2016). When collecting samples from a site, contamination of the water could lead to false positive detections. Fish, such as koi carp, release DNA into the environment, for example from sloughed skin cells (Avery, Humphrey, Keacher, & Bruce, 2014; Banks & Hogg, 2015; Goldberg et al., 2013; Knox et al., 2008). Some of this eDNA may be successfully detected through eDNA analysis, indicating the presence of koi carp. However, DNA can be present in an environment from sources other than live target organisms (Hinlo et al., 2017). For example, Knox et al. (2008) sequenced eel DNA from goldfish faeces. Contamination of an environment with koi carp DNA could occur through the presence of a koi carp carcass or excreted koi carp DNA, and this DNA contamination could persist in the environment for some time (Goldberg et al., 2013; Hinlo et al., 2017). When analysing low concentrations of eDNA, false positive detections can also occur from contamination during the laboratory processing of samples or a lack of primer specificity, such as overlapping DNA sequences with non-target, closely related species (Bylemans, Furlan, Pearce, Daly, & Gleeson, 2016; Furlan et al., 2016; Furlan & Gleeson, 2017; Goldberg et al., 2013; Herder et al., 2014).

Every detection tool has its limitations and eDNA is no exception (Hinlo, Gleeson, et al., 2017). eDNA methods are used in New Zealand (Jeunun et al., 2019); however, the development of alternative detection methods is required (Grainger, 2015a). Dog scent detection may be able to address the need for a feasible, reliable and adequately sensitive approach to the detection of invasive aquatic species.

Dog scent detection methodological difficulties

In dog scent detection research and operational scenarios, often a dog and handler team are utilised. Owning or leasing a scent detection dog or employing a dog and its handler can be expensive and may not be a cost-effective method in some situations (Gsell et al., 2010; Long et al., 2007; O'Connor, Park, & Goulson, 2012). For example, in New Zealand, the employment of a rodent scent detection dog and its handler can cost more than 300 NZD per day (Gsell et al., 2010). Not only can it be costly, but the presence of a handler can inadvertently affect the reliability of a scent-detection dog (Alexander et al., 2015). For example, if a handler knows the location of a target, they may affect scent detection outcomes through unintentional body language, correctly or incorrectly, cueing the dog (Alexander et al., 2015; Lit, Schweitzer, & Oberbauer, 2011).

In controlled conditions, diligence in the preparation and set up of samples is essential to avoid contaminating target samples with any other scent. Due to dogs' olfactory sensitivity, the scent of a handler or an experimenter's gloves can provide additional cues to dogs, altering detection rates (Alexander et al., 2015; Cooper, Wang, & Singh, 2014). Away from a controlled setting, the performance of a scent-detecting dog may be difficult to maintain. In field conditions, a target scent may be diluted by a myriad of other scents present in the environment, increasing the difficulty of the task. If no targets are present or found at the search location, the dog’s performance may wane, and if a scent-detection dog is not working effectively, then findings are inaccurate (Cablk & Heaton, 2006; Cooper et al., 2014; Porritt et al., 2015). Environmental conditions, such as wind direction and speed, can also affect the dispersal of target scent making pinpointing the target’s location difficult, as was found with dogs trained to detect the invasive brown marmorated stink bug (*Halyomorpha halys*) in woodlands, and invasive brown tree snakes (*Boiga irregularis*) in forested areas of Guam (Lee, Cullum, Anderson, Daugherty, Beckett & Leskey, 2014; Savidge et al., 2011).

It is important to consider the drawbacks of using dogs in scent detection applications and to conduct dog scent detection research in a way that improves the feasibility of the approach and reliability of the findings.

The present investigation

Terrestrial and aquatic habitats are vulnerable to incursions from species that evade border control and from the wider dispersal of localised, already established populations of invasive species (Koehn, 2004; Strayer, 2010). Dogs have been utilised in the detection of various organisms, and their detection capabilities have been shown to be suitably sensitive with high levels of accuracy across multiple trials and with very low numbers of targets (DeShon et al., 2016; Goodwin et al., 2010; Gsell, et al., 2010; Hoyer-Tomiczek et al., 2016; Mendel et al., 2018; Pfiester et al., 2008; Suma et al., 2014; Ward et al., 2014).

This research utilised a similar procedure and some of the same subjects as those used in research by Quaife (2018) and Crawford (2018). Quaife (2018) found that dogs trained to detect the scent of koi carp from sampled aquaria water could detect the fish-related VOCs and discriminate between target (koi carp) and non-target (no-fish, goldfish) water samples calculated at an effective biomass of 37.4 kg/ha. Leading on from this research, discrimination of non-target (no-fish, goldfish) from target (koi carp) samples was observed, but with variable performance, at an effective biomass of 18.7 kg/ha (Crawford, 2018).

The objective of this research was to investigate the scent-detection accuracy of dogs as they learned to discriminate between water samples from aquaria containing koi carp, goldfish or no fish, at systematically diluted concentrations.

The findings of this research will contribute to the development and evaluation of methods involving scent-detection dogs for koi carp detection.

Chapter 2: Method

Ethics

Ethical approval of this study was obtained from the University of Waikato Animal Ethics Committee (Protocol 1013).

Recruitment of dogs

Dogs were recruited through internal advertising at the University of Waikato and by word-of-mouth. A recruitment advertisement was also submitted to local dog groups' social media pages. Initial enquiry forms (Appendix A) detailing the recruitment criteria for potential research subjects, and consent forms (Appendix B) were sent by the researcher to all dog owners who expressed interest in the research. Owners were given the opportunity to ask any questions, and those interested in having their dog(s) participate in the study were asked to return the completed initial enquiry form and sign the consent form.

Inclusion/exclusion criteria

Upon receiving the completed initial enquiry and signed consent forms, a time was arranged for each owner whose dog met the initial criteria to visit the Canine Research Facility to further assess the suitability of their dog for the study. Selection criteria were based on behavioural tendencies and food motivation. Dogs that displayed relaxed body language at the facility without their owner present and showed continued interest in working for a food reward were included in the study. Any dog displaying signs of aggression or ongoing fear and distress was excluded from participating in the research. Attendance at the Canine Research Facility at specific times, on particular days, was also required. Eight recruited dogs were deemed unsuitable and were excluded from participating in experiments (Table 1). Subject Sabi was recruited mid-year and did not complete the training in time to participate in this study.

Table 1

Details of the Dogs Recruited for This Study

Subject	Breed	Sex	Age (years)	Selection criteria met	Fish scent discrimination training before study	Experiment participation
Mica	Blue heeler/heading x	Bitch	6	Yes	1 year	Yes
Luna	Border collie x	Spayed Bitch	4	Yes	1 year	Yes
Ruby	Labrador retriever/border collie x	Spayed Bitch	6	Yes	11 months	Yes
Sabi	Labrador	Spayed Bitch	5	Yes	0	No
Chobe	Labrador	Spayed Bitch	2	No	0	No
Nova	Crossbreed	Spayed Bitch	2	No	0	No
Pringle	Pembroke corgi	Spayed Bitch	0.9	No	0	No
Pip	Crossbreed	Spayed Bitch	2.5	No	0	No
Rosie	Cocker spaniel	Spayed Bitch	3	No	0	No
Kikko	Whippet x	Neutered Dog	1.2	No	0	No
Jack	Pitbull/great dane x	Neutered Dog	0.11	No	0	No
Ruby	German shepherd	Bitch	3.5	No	0	No

Subjects

Dogs.

Subjects were three female dogs of mixed breeds, ranging from 4 to 6 years old when the research began (Table 1). All three dogs had experimental experience in the specific scent discrimination task. Being privately owned, the dogs were housed at the facility only on experimental days (Thursdays and Fridays), between 7:30 am and 5:30 pm. When not participating in experiments, the dogs were housed individually in crates (1.8 m long x 1.2 m wide) that contained bedding and a water bowl. The weight of each dog was monitored throughout the experimental period (approximately 9 months). Water was available at all times and refreshed during the day. The dogs were periodically exercised outside the facility in a grassy field. Practices of care were conducted in accordance with the relevant Standard Operating Procedure (Handling and Care of Pet Dogs for Research) approved by the animal ethics committee.

Fish.

One koi carp and three goldfish were housed separately in two aerated fibreglass aquariums (59 cm high x 72 cm long x 81 cm wide) containing dechlorinated water. The room was air-conditioned, maintained at 17˚C, and lit on a 12-h light/dark cycle. Fish were fed weekly with a commercial fish food, and feeding amount was adjusted according to their weight (koi carp: range 1.748 – 1.821 kg; goldfish: range 0.687 – 0.745 kg) and the season. Practices of care were conducted in accordance with the relevant Standard Operating Procedure (Captive Handling and Captive Maintenance of Fish) approved by the animal ethics committee.

Experimental setting

Experiments took place at the Canine Research Facility on the University of Waikato main campus. There were five separate rooms in this building. An office and a kitchen were located at the front of the building. In the kitchen, there was a bench for sample preparation and a fridge for sample storage. The central room of the building was where the dogs were housed. This room also contained the computers used to collect the experimental data, and the researcher was positioned in this room during experimental sessions. The experimental room (3.2 x 4.3 m) was located at the back of the building with the apparatus set up in the corner. A thermometer was attached to the wall above the apparatus, and an air conditioning unit was positioned on the opposite wall. Next to the experimental room was a restroom.

Apparatus

The apparatus (1 m^3) contained 17 removable stainless-steel segments on a rotating carousel (Figure 1). Each segment was 280 mm tall, 135 mm across the front, 80 mm across the back, 145 mm deep, and had a volume of 3.57 L. Samples were centrally positioned on the lower plate of the carousel within each segment. A lid was placed on top of the segments creating an enclosed space around each sample (Figure 2). Across the front side, each segment had a hinged metal flap (100 mm square set, positioned 30 mm from the top, 100 mm from the bottom) that moved inwards when a sample was sniffed by a dog and back into a closed position when the dog retracted its nose.

Access to an individual segment and sample was through a 10-cm port on the front panel of the apparatus (Figure 3). A tone (approximately 3.7 kHz) signalled when an infrared beam across the port was broken, automatically recording response durations. On the right-hand edge of the front panel, an omnidirectional limit switch (Figure 3), when triggered, rotated the apparatus to the next segment and sample.

An automated feeder (PetSafe Treat & Train™ Remote Reward Dog Trainer) was positioned 1.5 m from the apparatus and reinforced correct indications with Pedigree® kibble. Two video cameras (Logitech® 2 MP HD Webcam C600) were positioned above the inside of the experimental room door. Session data and videos were recorded by custom-made software on two computers (Dell™ Optiplex™ 780) located in the adjacent room. The delivery of reinforcements, data collection and sample presentation was automated. Session data were also recorded manually on data sheets.

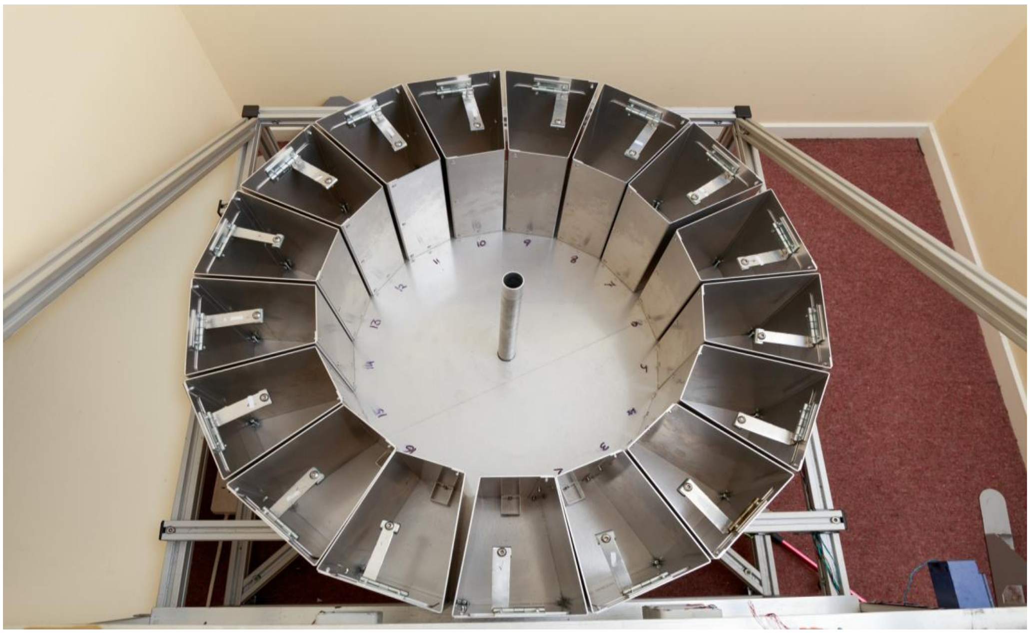

Figure 1. Scent-detection apparatus showing segments (1 to 17) positioned on the lower plate of the carousel with the apparatus lid removed.

Figure 2. Scent-detection apparatus with the lid positioned on top of the segments.

Figure 3. Front panel of the scent-detection apparatus showing the port (centre of front panel) and limit switch (right edge of front panel).

Aquatic research centre

The fish aquaria were located in the Aquatic Research Centre on the University of Waikato main campus. Three aquaria, situated side by side, were used in this study. Each contained a hose and an airstone bubbler on the end of a plastic aeration tube. One aquarium did not contain any fish, and water from this aquarium was used as the control sample for experiments. This aquarium had the same dimensions and composition as the aquariums containing fish, as described in section *Fish*.

Sample preparation

To prepare for sample collection, aquariums were cleaned on Wednesday mornings, between 5:30 and 6:30 am. The control (no-fish) aquarium was always cleaned first, followed by the goldfish aquarium, and then the koi carp aquarium. This order ensured that the positive sample was always handled last, reducing any risk of cross-contamination.

The cleaning procedure involved draining the water, scrubbing the aquarium surfaces with a scouring pad, and rinsing all aquarium surfaces. The fish remained in the aquariums during the cleaning procedure; the water was drained to a minimum level at which the fish were still fully covered by water. Gloves were worn and changed between each aquarium, and scouring pads designated to each aquarium were used.

Usually the water flow to each aquarium was left on at a slow, steady rate; however, to obtain sufficient fish-related volatiles in the sample water, the goldfish and koi carp aquariums were refilled to 50 L and 115.2 L, respectively, and the water flow turned off. These sampling volumes were based on the weight of the fish (kg) per litre of water and were used to obtain approximately equal concentrations of fish-related volatile organic compounds in the samples taken from the aquarium water. The fish aquarium water was left standing for 24 h before sample collection. The control aquarium water flow remained on at a slow, steady rate. The cleaning and preparation procedure was repeated on Thursday mornings for samples to be collected on Fridays (Appendix C).

Sample collection

Samples were collected on Thursday and Friday mornings. Samples were always taken from the control (no-fish) aquarium first, followed by the goldfish aquarium, and then the koi carp aquarium. Gloves were worn and changed between the handling of each sample type.

A beaker was used to fill two 2-L amber glass Winchester bottles with water from the control aquarium. Once full, the bottles were placed in a Winchester carrier for transit to the Canine Research Facility. A second beaker was used to partially fill a 1.5-L clear glass Winchester bottle with sample water from the goldfish aquarium. A third beaker was used to partially fill a 2-L, amber glass Winchester bottle with the koi carp aquarium water. The goldfish and koi carp sample bottles were placed in large plastic Glad® Snap® Lock bags and put in a backpack for transport to the Canine Research Facility. Each beaker was washed and stored separately. After samples were collected for experimental sessions on Friday mornings, the water flow of the aquariums containing fish was turned back on and set at a slow, steady rate (Appendix C). Samples were stored in a fridge at 4°C during the day at the Canine Research Facility.

Cleaning of sample collection bottles

The Winchester bottles were washed weekly in hot water mixed with detergent and rinsed. The control (no-fish) bottles were always cleaned first, followed by the goldfish bottle and lastly the koi carp bottle. The basin was rinsed with boiling water and refilled between the washing of each bottle. All bottles were tipped upside down and left to air dry in separate locations in the same room.

Sample set-up

Samples were prepared in 200 g clear glass jars (89 mm diameter x 60 mm height). Seventeen glass jars were separated into three groups; five for non-target (no-fish) samples, five for non-target (goldfish) samples and seven for target (koi carp) samples. A small white sticker was placed horizontally on the underside of the non-target (goldfish) sample jars and placed vertically on the outer side of the target (koi carp) sample jars. Apparatus segments were allocated to a specific sample type. Therefore, non-target (goldfish) and target (koi carp) apparatus segments also had a corresponding horizontal or vertical sticker affixed at the top of the segment. This allowed for easy identification and arrangement of sample and segment types.

From one of the control (no-fish) sample Winchester bottles, 100 mL was syringed into each of the 17 glass sample jars. The non-target (no-fish) samples were then placed on the apparatus in a prearranged randomised order, and allocated control segments were placed over the top of each sample. Next, from the goldfish sample bottle, approximately 100 mL was poured into a spare glass sample jar. An auto-pipette was used to deliver a predetermined amount (see *Experimental sessions* section) of the goldfish aquarium water into each of the non-target (goldfish) sample glass jars. The goldfish sample jars were then placed on the apparatus base, and an allocated goldfish segment placed over each sample. Lastly, about 100 mL from the koi carp sample bottle was poured into another spare glass sample jar. An auto-pipette delivered the same predetermined amount of koi carp aquarium water into each of the target (koi carp) sample glass jars. The target (koi carp) sample jars were then placed on the apparatus base, and an allocated koi carp segment was placed over the top of each sample. Once all samples and segments were positioned on the apparatus, the apparatus lid was placed on top of the segments. The samples were left in the apparatus for at least 15 min before sessions began.

After each set of experimental sessions, the segments and samples were removed (no-fish first, then goldfish, followed by koi carp) and placed in segregated locations within the experimental room. The base of the apparatus and underside of the apparatus lid were cleaned with a 70% isopropanol $(CH^3)^2CHOH$ solution. Once the apparatus was clean and dry, the next randomised arrangement of samples was set up for the next set of sessions. Gloves were worn at all times and changed between sample and segment types.

The samples were refreshed at about midday. This was done to replenish the fish-related volatile organic compounds that may have evaporated during the morning sessions. The used samples were tipped into the kitchen sink and new samples were taken from the sample Winchester bottles.

At the end of experimental sessions each day, the apparatus base and lid were cleaned with a 70% isopropanol solution. Used sample glass jars were submerged in concentrated (65%) HNO_3 for at least 24 h, then rinsed with distilled water and placed in a Contherm Thermotec 2000 oven, between 70 and 100°C. If segments were to be used again the next day, then they were not washed but left in their segregated locations within the experimental room. On the last day of experimental sessions, the segments were washed in warm water mixed with detergent and left to air dry.

Experimental sessions

Up to seven sets of experimental sessions were run per day, two days per week, between 8 am and 4:30 pm. The 17 samples were presented to each dog twice per session (34 trials) with each dog taking 3 to 5 minutes to complete one session. A go/no-go procedure was used in this study; dogs were trained to respond to target (koi carp) samples for a minimum indication time (3,501 ms to 5,501 ms, different for each dog) and required to assess non-target (no-fish and goldfish) samples for at least 501 ms and then trigger the limit switch to gain access to the next trial (see Quaife, 2018, for the training procedure of this study's subjects). Positive indications (hits) were reinforced with one piece of kibble. There were no consequences for correct rejections, misses or false indications.

The dilutions of the sampled aquaria water used in this study were converted into effective biomass densities (Table 2) based on the calculations described by Quaife (2018). Each effective biomass density was calculated by dividing the sample dilution (e.g., 0.19) by 0.098 (*dilution from Quaife, 2018*) and multiplying the quotient by the effective biomass density (*37.4 kg/ha from Quaife, 2018*). Effective biomass = (dilution/ 0.098) x 37.4.

Table 2

Experimental Dilutions (mL/100 mL) and Effective Biomass Densities (kg/ha)

Dilution	Biomass
0.19	72.5
0.098	37.4
0.049	18.7
0.024	9.2
0.015	5.7
0.012	4.6
0.0075	2.9
0.006	2.3
0.00375	1.4
0.003	1.1
0.00188	0.7
0.0015	0.6
0.00094	0.4

Experimental sessions began with the dogs discriminating between samples at a dilution of 0.049 mL/100 mL. These initial experimental sessions were conducted to establish a baseline level of dog performance. If performance was observed to be quite variable, then response durations were manipulated. Therefore, the response duration for two of the subjects (Mica and Luna) was manipulated during this time resulting in respective minimum positive indication times of 4,001 and 5,501 ms. Responding by subject Ruby stabilised early on in experimental sessions; therefore, no positive indication increase was required and her indication time remained at 3,501 ms. Indication times stayed the same for the remainder of the experimental sessions. A criterion of four consecutive sessions at an 80% hit and correct rejection rate was set. Once responding for all three subjects had stabilised at the 80% criterion, the dilution was halved (rounded down to accommodate the auto-pipette scale) to 0.024 mL/100 mL.

Control test

Rapid acclimation (i.e., performance at criterion) to samples at a dilution of 0.024 mL per 100 mL indicated there may be extraneous variables influencing indication rates. To establish whether this was the case, a control test was performed with subjects Mica and Luna. Subject Ruby was not available to participate in experimental sessions at this time. When preparing the dilutions, the same dilution procedure as mentioned in *Sample set-up* was followed; however, all samples contained only control (no-fish) sample aquarium water so that any potential influence of the dilution procedure could be evaluated. Allocated target (koi carp), and non-target (goldfish and no-fish) sample segments had been washed and were used as described in *Sample set-up*.

Modifications to segments and cleaning procedure

It was expected that correct rejection responses in the control test would be distributed approximately evenly among all samples and segments. However, it appeared extraneous variables were influencing the dogs' performance. Following the control test, new measures were put in place to assess possible extraneous variables and to re-establish stimulus control. Firstly, the small white stickers on the top of the segments were removed, as it was considered possible that sample scent may have become trapped there. Secondly, segments were no longer allocated to a sample type, instead between days, segments were unsystematically selected to contain any sample. The same segments were used for non-target (no-fish), non-target (goldfish) and target (koi carp) samples in subsequent sessions within an experimental day. This was due to sample odour accumulating on the inside walls of the segments. Quaife (2018) had initially randomised segments to sample type each session but found that contamination was occurring. Also, it was an inefficient practice to clean the segments between sessions.

Thirdly, the cleaning procedure of the segments was modified in case sample residue was not being removed effectively (e.g., remained in the grooves of the segments). One Sunlight Power Max® dishwashing tablet containing enzymes, was dissolved in 10 cm of warm water in the kitchen sink. The segments were washed in the sink and rinsed with warm water. Each segment was then dipped in a solution of 50/50 isopropanol $(CH^3)^2CHOH$ and water and left to air dry. Segments were washed every day after sessions, and this cleaning procedure continued for the remainder of the experimental days.

Dilution and segment change

The sample dilution was changed from 0.024 mL/100 mL to 0.098 mL/100 mL; a dilution at which the dogs previously met the criterion within 14 sessions (Quaife, 2018). At this dilution (0.098 mL/100 mL), a new set of segments was introduced, identical in dimensions to the previous set, and was used for the remaining experimental sessions.

Due to the dogs’ performance not improving rapidly with samples at a dilution of 0.098 mL per 100 mL, the sample dilutions were increased (0.19 mL per 100 mL) to establish stimulus control by the fish-related volatile organic compounds.

Multiple-probe design

Once higher levels of performance were occurring, a multiple-probe design experiment was conducted to assess scent-detection thresholds at multiple dilutions. Three sample jars were taken from the non-target (no-fish) group and used for probe samples. Therefore, only two samples out of 17 were non-target (no-fish) samples in experimental probe sessions.

The probe samples were prepared after the non-target (goldfish) samples and before the target (koi carp) samples (see *Sample set-up*). The procedure included placing a small white sticker horizontally on the bottom outer edge of each probe sample glass jar. Probe samples were prepared from highest to lowest dilution and a new pipette tip was used for each dilution. A spare glass sample jar was filled with 100 mL of target (koi carp) aquarium sample water. An auto-pipette was used to deliver target (koi carp) sample water from the spare jar to each probe sample jar, at one of the specified dilutions (Table 3). Serial dilutions were prepared for 0.0015, 0.00375, 0.00188, and 0.00094 mL per 100 mL probe samples. The seven target (koi carp) and five non-target (goldfish) sample jars were always prepared at a dilution of 0.19 mL per 100 mL.

The probe samples were positioned on the apparatus after non-target (goldfish) samples and before target (koi carp) samples, in a prearranged randomised order, and covered with a randomly selected segment. The same segments were used for each probe sample in subsequent sessions within an experimental day. When samples were re-randomised, probe samples were removed in order from highest to lowest dilution and placed segregated from all other sample and segment types.

Positive indications (hits) on probe samples were not reinforced. Three probe samples with variable dilutions were tested per experimental day. In an experimental day, if the dogs' hit rate was higher than their false indication rate on a set of three probe samples, then the dilutions were increased (concentration reduced) the next experimental day. Due to human error, dilutions of probe samples 13, 14 and 15 were decreased slightly from the previous sample dilutions of probes 10, 11 and 12. Dilutions were increased in the following set of three probe samples (16, 17 and 18).

Table 3

Multiple Probe Design Dilutions (mL) per 100 mL

Probe (1, 2, 3)	0.098	0.049	0.024
Probe (4, 5, 6)	0.049	0.024	0.012
Probe (7, 8, 9)	0.024	0.012	0.006
Probe (10, 11, 12)	0.006	0.003	0.0015
Probe (13, 14, 15)	0.015	0.0075	0.00375
Probe (16, 17, 18)	0.00375	0.00188	0.00094

Session with no samples

To explore the accumulation of volatile organic compounds within segments, a session with no samples was conducted. Seven sets of sessions were run with two dogs. Samples were not refreshed during the day. For the seventh session, all sample glass jars were removed from the experimental room. The segments that had been used for non-target (no-fish), non-target (goldfish) and target (koi carp) samples in the previous six sets of sessions were arranged in a prearranged randomised order on the apparatus and each dog was run through one session.

Data analyses

Individual visual analyses of data collected across experimental, multiple probe and no sample sessions were conducted. Due to an observed pattern of first trial positive indicating over the course of the data collection period, the proportion of correct indications on first trials, calculated as sensitivity, specificity and overall accuracy, were compared with those of second trials.

Chapter 3: Results

Experimental data

Figures 4-9 show the sequential performance of each dog, from the beginning of the experimental sessions, across four different dilutions, segment changes (i.e., segment randomisation and new segments), and multiple probe sessions. The percentage of hits on target (koi carp) samples and correct rejections of non-target (goldfish and no-fish) samples are presented, per session, in Figures 4, 6 and 8. Figures 5, 7 and 9 show the percentage of hits on target (koi carp) samples and the combined correct rejections of non-target (goldfish and no-fish) samples, per session.

Figure 4 shows the performance of subject Mica. At a dilution of 0.049 mL per 100 mL, a relatively stable, high level of performance was observed for target samples with accuracy rates ranging from 79% to 100% (*M* = 96.9%, *SD* = 5.9%). Mica's performance showed more variability with non-target samples ranging from 40% accuracy to 100% accuracy (*M* = 87.1%, *SD* = 12.6% for goldfish samples, *M* = 90.9%, *SD* = 13.0% for no-fish samples). Mica's response duration was manipulated several times during experimental sessions at a dilution of 0.049 mL per 100 mL. Therefore, the next experimental phase was not introduced until performance was observed to be relatively stable (i.e., when most accuracy rates were at or above 80%). Thus, out of a total of 78 sessions, the criterion (four consecutive sessions at or above 80% accuracy for all sample types) was met nine times, including in the last four experimental sessions (Figure 4, sessions 75 to 78) before progression to the next experimental phase.

With a decrease in concentration (to 0.024 mL/100 mL), performance was initially quite variable with accuracy rates ranging from 64% to 93% for target samples and 50% to 100% for non-target samples, in the first four sessions. For the remainder of sessions, Mica's performance shows relative stability with no less than 70% accuracy and up to 100% accuracy for all sample types, meeting the criterion three times. On average, overall accuracy rates were above criterion levels (*M* = 91.5%, *SD* = 10.4% for koi carp samples, *M* = 91.6%, *SD* = 13.8% for goldfish samples, *M* = 88.4%, *SD* = 15.9% for no-fish samples).

Potential confounding factors were assessed by conducting one session with all non-target (no-fish) samples. Up until the control test, specific segments were allocated to a sample type. The percentage of hits on segments used exclusively for target (koi carp) samples and the correct rejections of segments used exclusively for non-target (goldfish and no-fish) samples are depicted by three solid grey data points in Figure 4. It was expected that all 17 washed segments containing non-target (no-fish) samples, would be correctly rejected and if any false alarms did occur, they would be approximately evenly distributed across segment types. Mica correctly rejected 100% of non-target (no-fish) segments containing control (no-fish) samples. False alarms occurred on 20% of non-target (goldfish) segments containing control (no-fish) samples and on 50% of target (koi carp) segments containing control (no-fish) samples. Five false alarms on target (koi carp) segments, out of a potential of seven, occurred during the first rotation of the samples; two occurred during the second rotation.

With the introduction of segment randomisation to sample type, an immediate change in performance was observed with the first session hit and correct rejection rates at less than 80% accuracy (Figure 4, session 105). The hit and correct rejection rates then increased, but as sessions progressed, they became highly variable, and a gradual decrease in accuracy was observed across sessions. On average, overall accuracy rates were below criterion levels (*M* = 78.6%, *SD* = 16.7% koi carp, *M* = 70%, *SD* = 22% goldfish, *M* = 73.3%, *SD* = 24% no-fish). After 18 sessions, the criterion had not been met.

An increase in concentration (to 0.098 mL/100 mL) was followed by somewhat less variability. The correct rejection rate of non-target (no-fish) samples was between 80% and 100% accuracy across sessions. However, hits on target (koi carp) samples and the correct rejections of non-target (goldfish) samples were below 80% accuracy in 47% of the sessions conducted during this experimental phase. After 15 sessions, the criterion had not been met.

With the introduction of new segments, accuracy measures increased steeply, decreased steeply, then increased again (Figure 4, sessions 138 to 145). With a further increase in concentration (to 0.19 mL/100 mL), a variable pattern of increasing and decreasing accuracies for all sample types was observed. By session 159, accuracy measures were more consistent in level and trend. The criterion was met in the last four sessions with hit and correct rejection rates at 80% to 100% accuracy.

For the multiple probe sessions, the number of non-target (no-fish) samples was reduced to two, with three probe samples (koi carp) replacing the three non-target (no-fish) samples. Hits on probe samples are not shown in Figures 4 and 5. During multiple probe experimental sessions, a recurring pattern was observed each experimental day. Hit and correct rejection rates generally began low (average first session accuracy, *M* = 72.9% for koi carp samples, *M* =

52% for goldfish samples, *M* = 57.5% for no-fish samples). As sessions progressed, accuracy rates steadily increased (average third session accuracy, *M* = 93.6% for koi carp samples, *M* = 95% for goldfish samples, *M* = 97.5% for no-fish samples) followed by relatively stable, high levels of performance in subsequent sessions within an experimental day. Out of a total of 45 sessions, the criterion (four consecutive sessions at or above 80% accuracy for all sample types) was met three times. On average, overall accuracy rates were above criterion levels (*M* = 89.1%, *SD* = 13.8% koi carp, *M* = 83.8%, *SD* = 22.8% goldfish, *M* = 89.4%, *SD* = 20.3% no-fish).

Figure 5 shows that across all experimental phases, Mica’s hit rates (96.9, 91.5, 78.6, 83.1, 87.8, 88.3 and 89.1%, respectively) were consistently higher than the combined correct rejection rates (89, 90, 71.7, 80.3, 73.8, 76.8 and 86.6%, respectively). In the control test, positive indications occurred on 50% of segments used for target (koi carp) samples, and correct rejections occurred on 90% of segments used for non-target (goldfish and no-fish) samples.

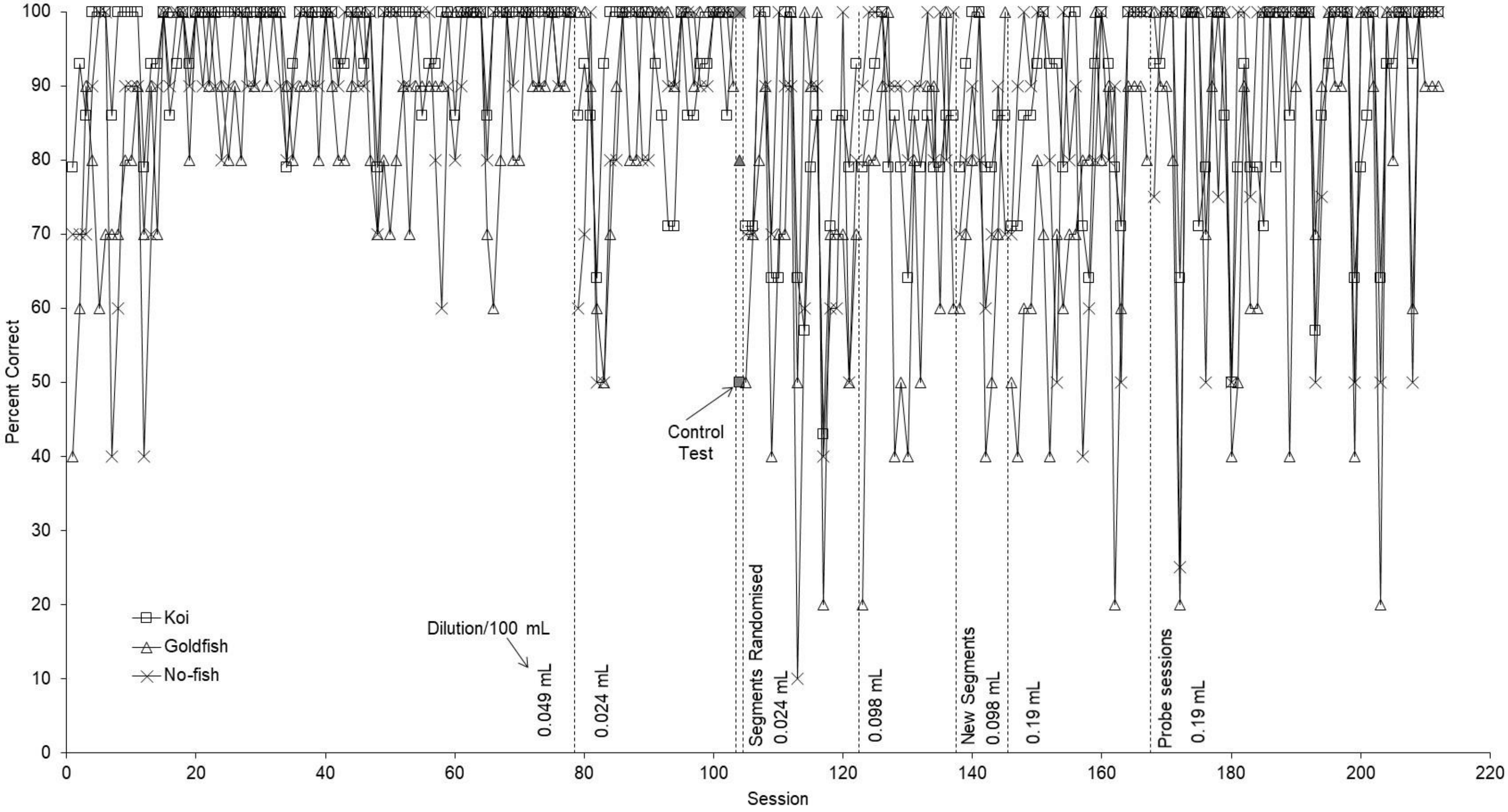

Figure 4. Mica’s percentage of hits on target (koi carp) samples and the correct rejections of non-target (goldfish, no-fish) samples, per session, across conditions. The three solid grey data points of the control test show the hits on target (koi carp) segments and the correct rejections of non-target (goldfish, no-fish) segments, when all samples contained only control (no-fish) aquarium water.

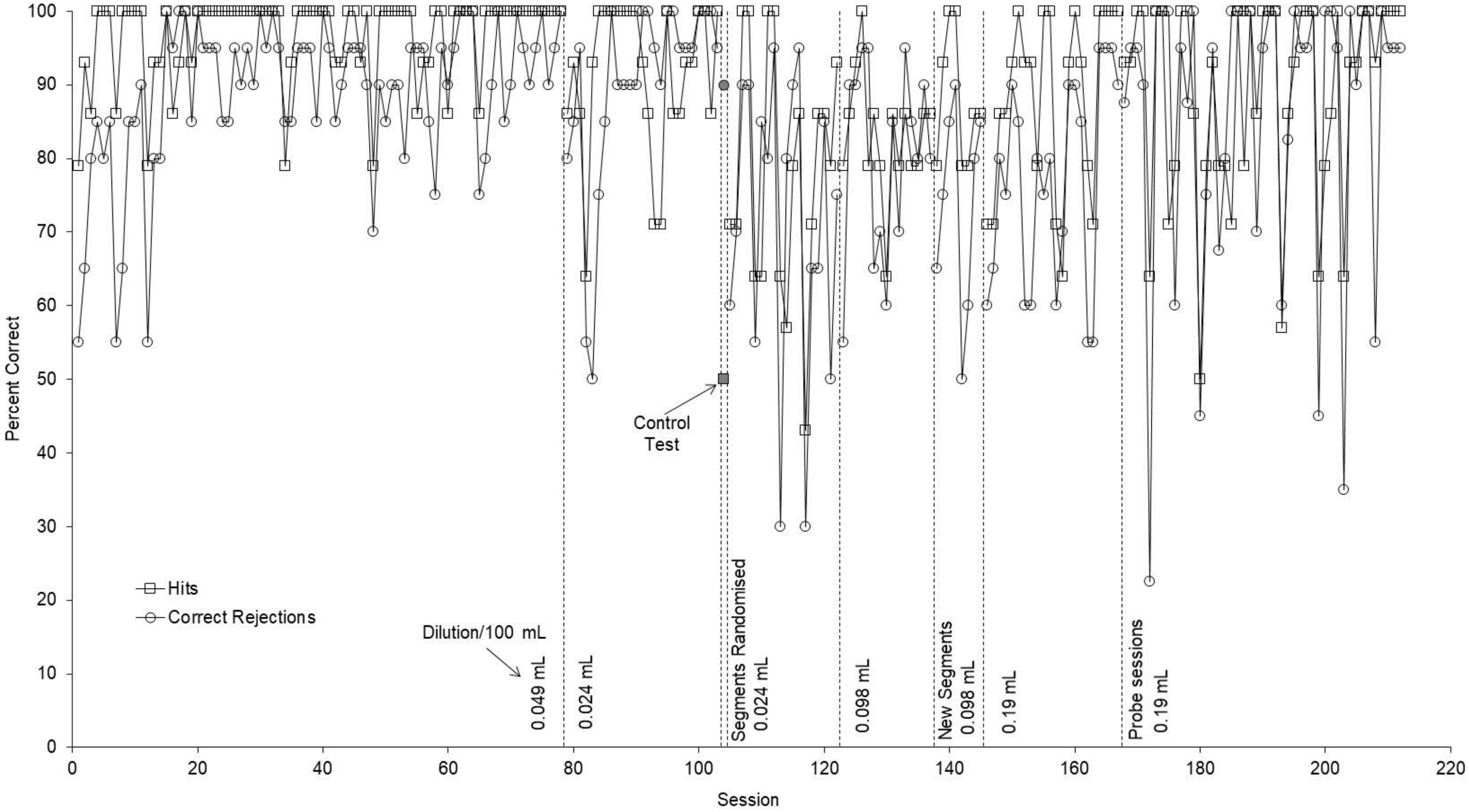

Figure 5. Mica’s percentage of hits on target (koi carp) samples and the combined correct rejections of non-target (goldfish, no-fish) samples, per session, across conditions. The two solid grey data points of the control test show the hits on target (koi carp) segments and the combined correct rejections of non-target (goldfish, no-fish) segments, when all samples contained only control (no-fish) aquarium water.

Figures 6 and 7 show the performance of subject Luna. Figure 6 shows at a dilution of 0.049 mL per 100 mL, correct rejection rates were occasionally highly variable, ranging between 10% and 100% accuracy for goldfish (*M* = 84.8%, *SD* = 18.4%) and 0% and 100% accuracy for no-fish (*M* = 83.4%, *SD* = 23.5%) samples. In contrast, hits on target (koi carp) samples were relatively stable and at a high level of accuracy across sessions (*M* = 97.8%, *SD* = 4.7%). Luna's response duration was manipulated several times during experimental sessions at a 0.049 mL per 100 mL dilution. Therefore, the next experimental phase was not introduced until performance was observed to be relatively stable (i.e., when most accuracy rates were at or above 80%). Thus, out of a total of 71 sessions, the criterion was met four times, including in the last four experimental sessions (Figure 6, sessions 68 to 71) before progression to the next experimental phase.

With a decrease in concentration (to 0.024 mL/100 mL), correct rejection rates were quite variable, and an overall, relatively low average accuracy rate was observed for no-fish samples (*M* = 82%, *SD* = 22.3% for goldfish samples, *M* = 69%, *SD* = 20.2% for no-fish samples). The hit rate remained stable and at a high level, in the range of 86% to 100% accuracy (*M* = 97.2%, *SD* = 4.9% for koi carp samples). Performance came close to meeting the criterion with three consecutive sessions between 80% and 100% accuracy for all sample types in the last three sessions.

Data recorded for the control test were incomplete and therefore are not presented in Figures 6 and 7. Luna completed only eight trials before lying down in the experimental room. The session was discontinued at this point. Indications occurred on 100% of target (koi carp) segments containing control (no-fish) samples, and correct rejections occurred on 50% of non-target (goldfish) segments containing control (no-fish) samples and on 50% of non-target (no-fish) segments containing control (no-fish) samples.

With the introduction of segment randomisation to sample type, hits on target (koi carp) samples and correct rejections of non-target (no-fish) samples were initially at a relatively high level of accuracy (93% -100% for koi carp samples, 70% - 90% no-fish for no-fish samples), whereas correct rejections of non-target (goldfish) samples showed high variability with accuracy ranging from 30% to 90%. A decrease in accuracy measures followed for all sample types. This trend gradually reversed; however, the criterion was not met. On average, overall accuracy rates were above criterion levels (80% accuracy) for target samples and below criterion levels for non-target samples (*M* = 90.8%, *SD* = 9.4% for koi carp samples, *M* = 55.2%, *SD* = 25.7% for goldfish samples, *M* = 69.1%, *SD* = 20.1% for no-fish samples).

With an increase in concentration (to 0.098 mL/100 mL), hits on target (koi carp) samples were stable at a high level (*M* = 96.5%, *SD* = 5.3%). A low level, fluctuating correct rejection rate was observed for non-target (goldfish) samples (*M* = 40%, *SD* = 23.9%) and correct rejections of non-target (no-fish) samples varied between high and lower levels of performance (*M* = 80%, *SD* = 27.8%). The criterion was not met.

When new segments were introduced, non-target accuracy rates increased from 60% (goldfish) and 70% (no-fish) accuracy, to 90% accuracy in the last experimental session. Following a further increase in concentration (to 0.19 mL/100 mL) correct rejection rates were highly variable for non-target samples (*M* = 64.5%, *SD* = 25.9% for goldfish samples, *M* = 75.3%, *SD* = 23.6% for no-fish samples). Hits on target (koi carp) samples were generally at a high level with moderate variability (*M* = 92%, *SD* = 10.7%). After 15 sessions, the criterion had not been met.

During the multiple probe experimental sessions, first session correct rejection rates generally began low (average first session accuracy, *M* = 38% for goldfish samples, *M* = 55% for no-fish samples) and steadily increased as sessions progressed (average third session accuracy, *M* = 82.5% for goldfish samples, *M* = 93.8% for no-fish samples) and were followed by relatively stable, high levels of performance in subsequent sessions within an experimental day. Hits on target samples followed a similar pattern but with far less variability (average first session accuracy, *M* = 96.5%, average third session accuracy, *M* = 100%). Out of a total of 41 sessions, the criterion was met once. On average, overall accuracy rates were above criterion levels for target and non-target (no-fish) samples (*M* = 98.3%, *SD* = 3.8% koi carp, *M* = 80.5%, *SD* = 27.1% no-fish) and just below criterion levels for non-target (goldfish) samples (*M* = 73.2%, *SD* = 28.3%).

Figure 7 shows Luna’s hit rate was higher (97.8, 97.2, 91.3, 96.5, 93, 92 and 98.3%, respectively) than the combined correct rejection rates (84, 75.5, 60.2, 60, 71.3, 70 and 76.8%, respectively) across all experimental phases.

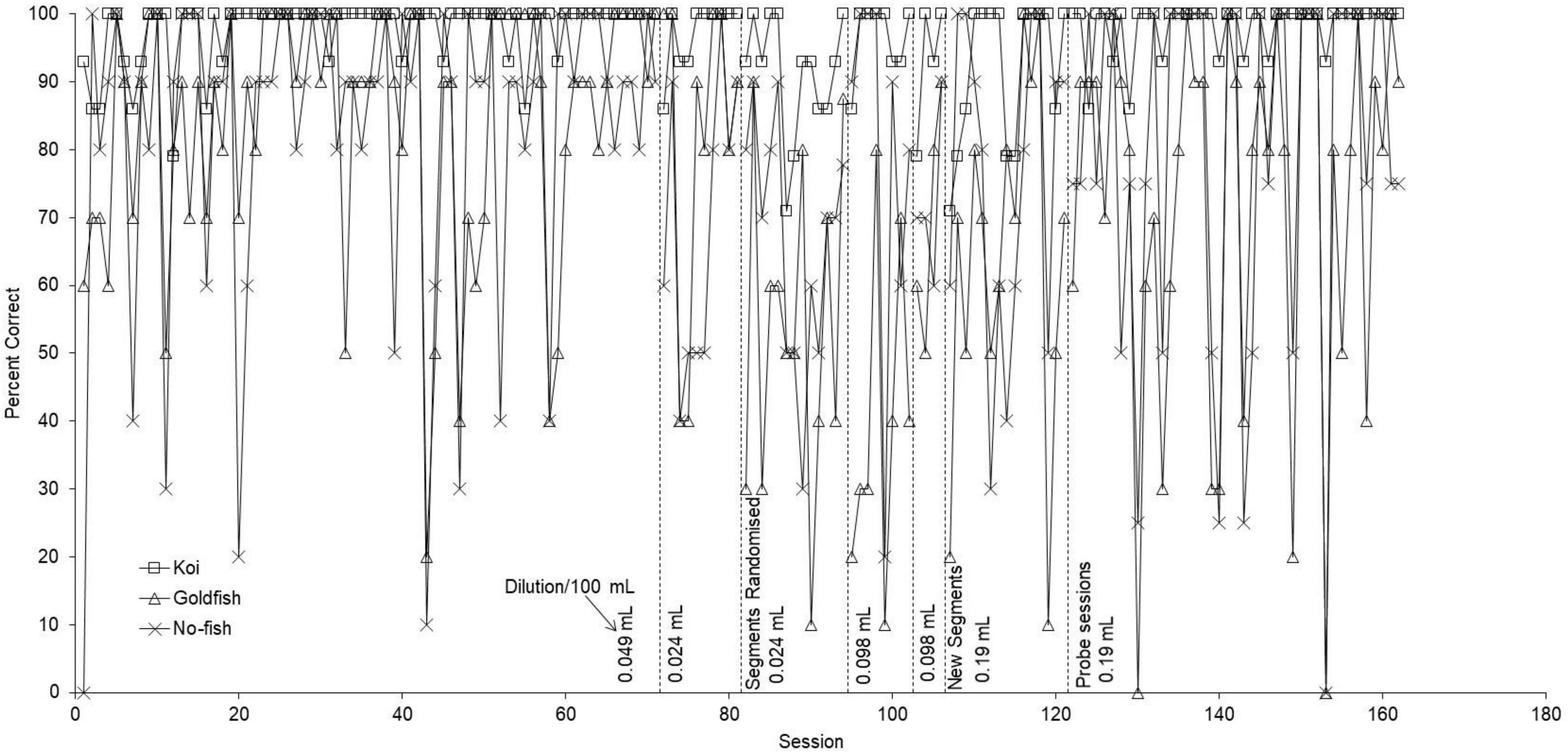

Figure 6. Luna's percentage of hits on target (koi carp) samples and the correct rejections of non-target (goldfish, no-fish) samples, per session, across conditions.

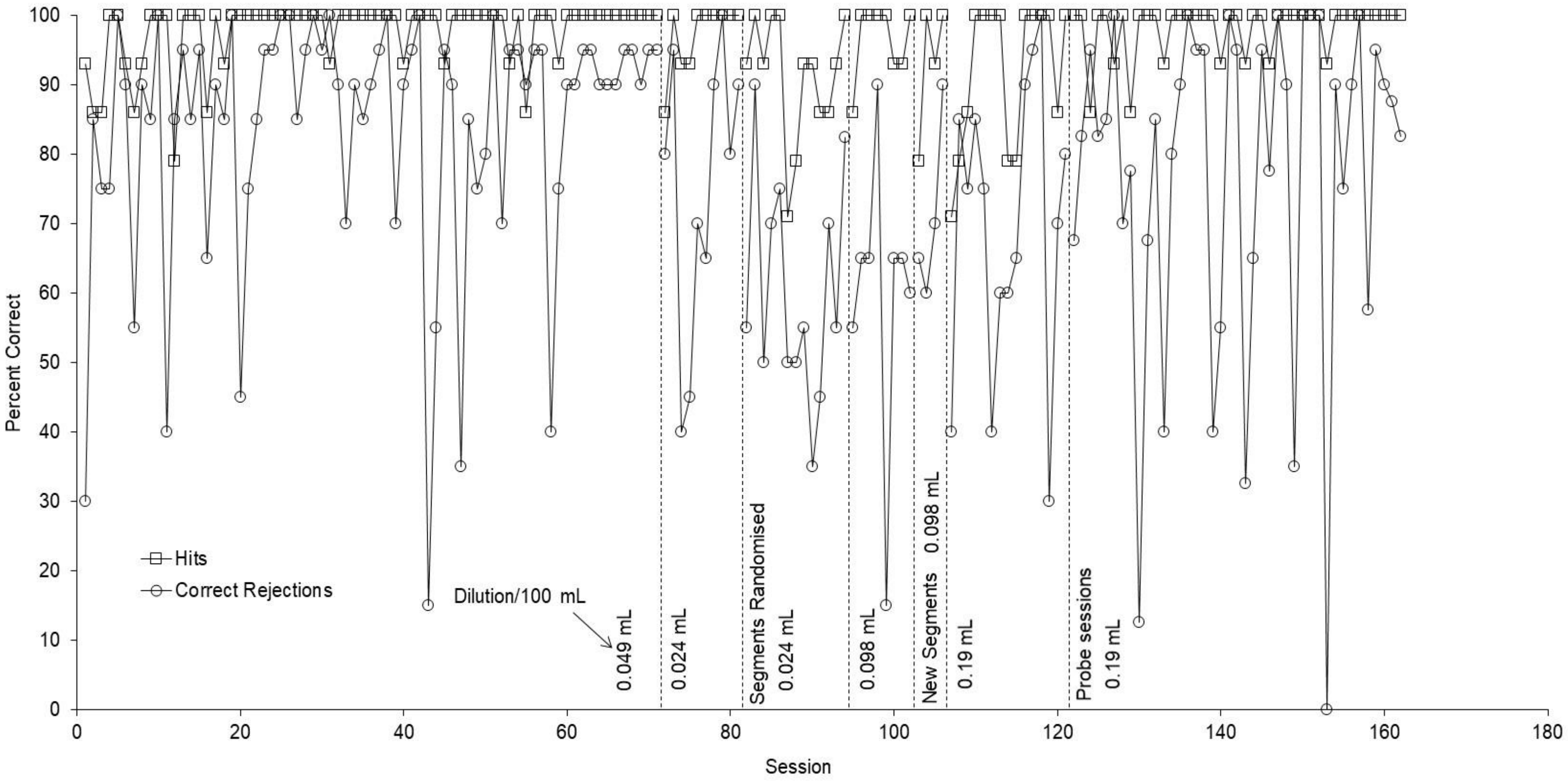

Figure 7. Luna's percentage of hits on target (koi carp) samples and the combined correct rejections of non-target (goldfish, no-fish) samples, per session, across conditions.

Figures 8 and 9 show the performance of subject Ruby. At a dilution of 0.049 mL per 100 mL, a stable, high level of performance was observed for target (koi carp) samples with accuracy rates between 86% and 100% (*M* = 98.9%, *SD* = 3.4%). Ruby's performance showed more variability with non-target samples ranging from 60% to 100% accuracy for goldfish samples (*M* = 91.4%, *SD* = 11.5%) and 40% to 100% accuracy for no-fish samples (*M* = 90.5%, *SD* = 13.3%). Out of a total of 44 sessions, as a baseline level of performance was established, the criterion (four consecutive sessions at or above 80% accuracy for all sample types) was met five times, including in the last four experimental sessions (Figure 8, sessions 41 to 44) before progressing to the next experimental phase.

With a decrease in concentration (to 0.024 mL/100 mL), performance remained stable at a high level with no less than 86% accuracy and up to 100% accuracy for all sample types, meeting the criterion twice (*M* = 97.5%, *SD* = 4.7% for koi carp samples; *M* = 96.4%, *SD* = 5.1%, for goldfish samples; *M* = 96.4%, *SD* = 5.1% for no-fish samples).

A control test was not conducted with Ruby. At the introduction of segment randomisation to sample type, an immediate decrease in accuracy measures was observed (40% to 71% accuracy). Accuracy measures then fluctuated between high and lower levels of accuracy. Correct rejections of non-target (goldfish) samples were highly variable ranging between 0% and 100% accuracy. On average, overall accuracy rates were at criterion levels for target (koi carp) and non-target (no-fish) samples and below criterion levels for non-target (goldfish) samples (*M* = 81.5%, *SD*

= 10.9% for koi carp, *M* = 68%, *SD* = 29% for goldfish, *M* = 81%, *SD* = 19.1% for no-fish samples). After 10 sessions, the criterion had not been met.

With an increase in concentration (to 0.098 mL/100 mL), correct rejections of non-target (no-fish) samples were relatively stable (*M* = 94%, *SD* = 7%) whereas correct rejections of non-target (goldfish) samples were more variable (*M* = 72%, *SD* = 16.9% goldfish). The hit rate fluctuated between 64% and 100% accuracy (*M* = 85.8%, *SD* = 11.3%). The criterion had not been met after 10 sessions.

With the introduction of new segments, accuracy rates occurred at a relatively high level across sample types (71% to 90% accurate). A change in concentration (to 0.19 mL/100 mL), shows a pattern of increasing and decreasing accuracies. On average, the overall accuracy rates were at criterion levels for target and non-target samples (*M* = 84.5%, *SD* = 13.3% for koi carp, *M* = 80%, *SD* = 16.6% for goldfish, *M* = 92.4%, *SD* = 10.3% for no-fish samples). The criterion was met in the last four consecutive sessions with hit and correct rejection rates between 80% and 100% for all sample types.

During the multiple probe experimental sessions, the hit rate decreased to a low level of 36% accuracy (Figure 8, session 100). Subsequent hits occurred at a high level ranging between 79% and 100% accuracy. The correct rejection rates were relatively variable ranging from 40% to 100% accuracy (goldfish) and 50% to 100% accuracy (no-fish). Out of a total of 12 sessions, the criterion was met once. On average, overall accuracy rates were above criterion levels for all sample types (*M* =

86.3%, *SD* = 19.6% for koi carp, *M* = 84.2%, *SD* = 19.3% for goldfish, *M* = 91.2%, *SD* = 16.3% for no-fish samples).

Figure 9 shows Ruby's hit rate (98.9, 97.6, 80.8, and 87.4%, respectively) was higher than the combined correct rejection rates (90.9, 96.4, 75.5, and 83.3%, respectively) across experimental phases until new segments were introduced (hits, 78.5% at 0.098 mL, 84.5% at 0.19 mL and 86.3% at 0.19 mL for probe sessions; combined correct rejections 82.5% at 0.098 mL, 86.2% at 0.19 mL and 87.9% at 0.19 mL for probe sessions).

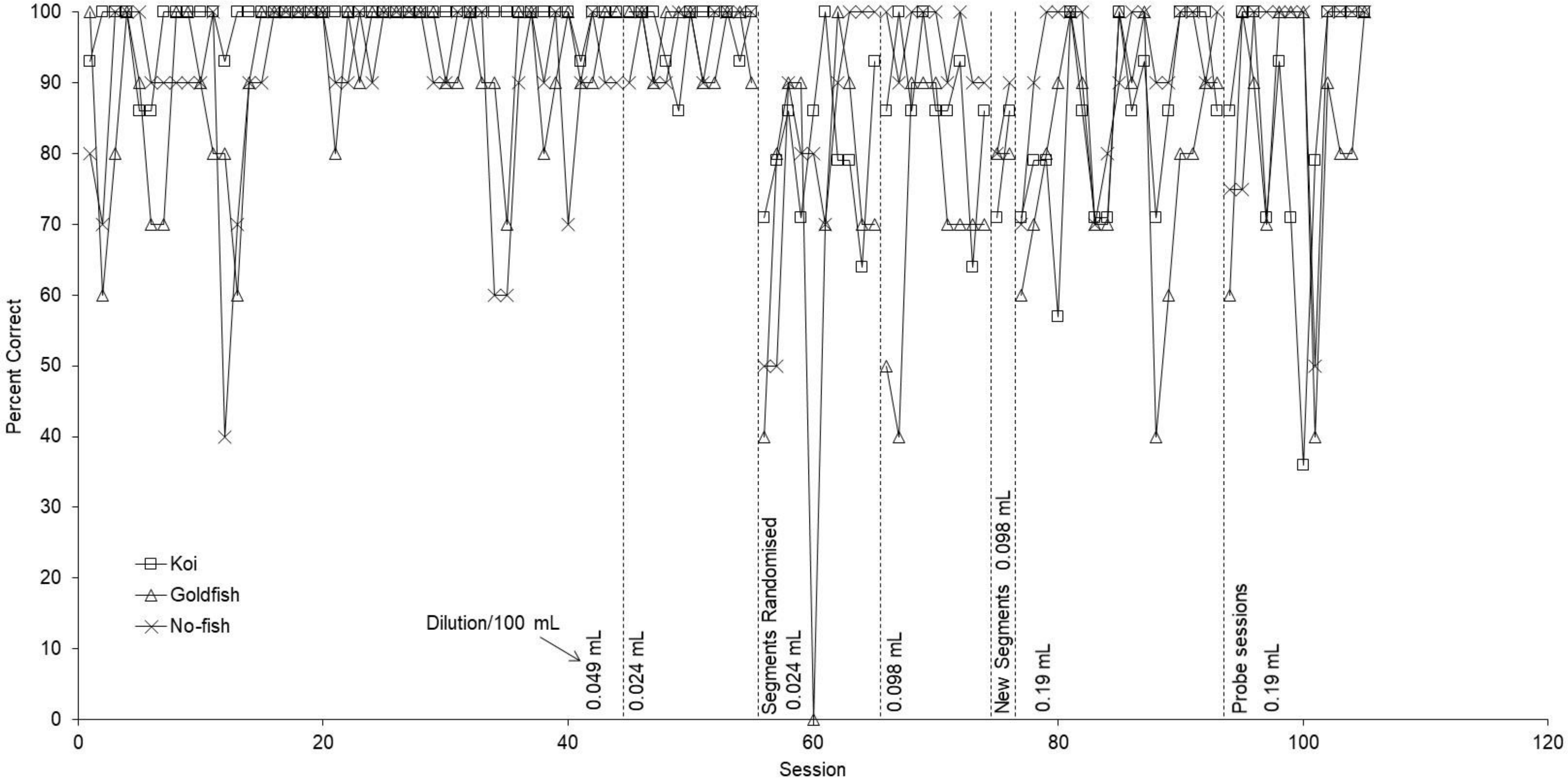

Figure 8. Ruby's percentage of hits on target (koi carp) samples and the correct rejection rates of non-target (goldfish, no-fish) samples, per session, across conditions.

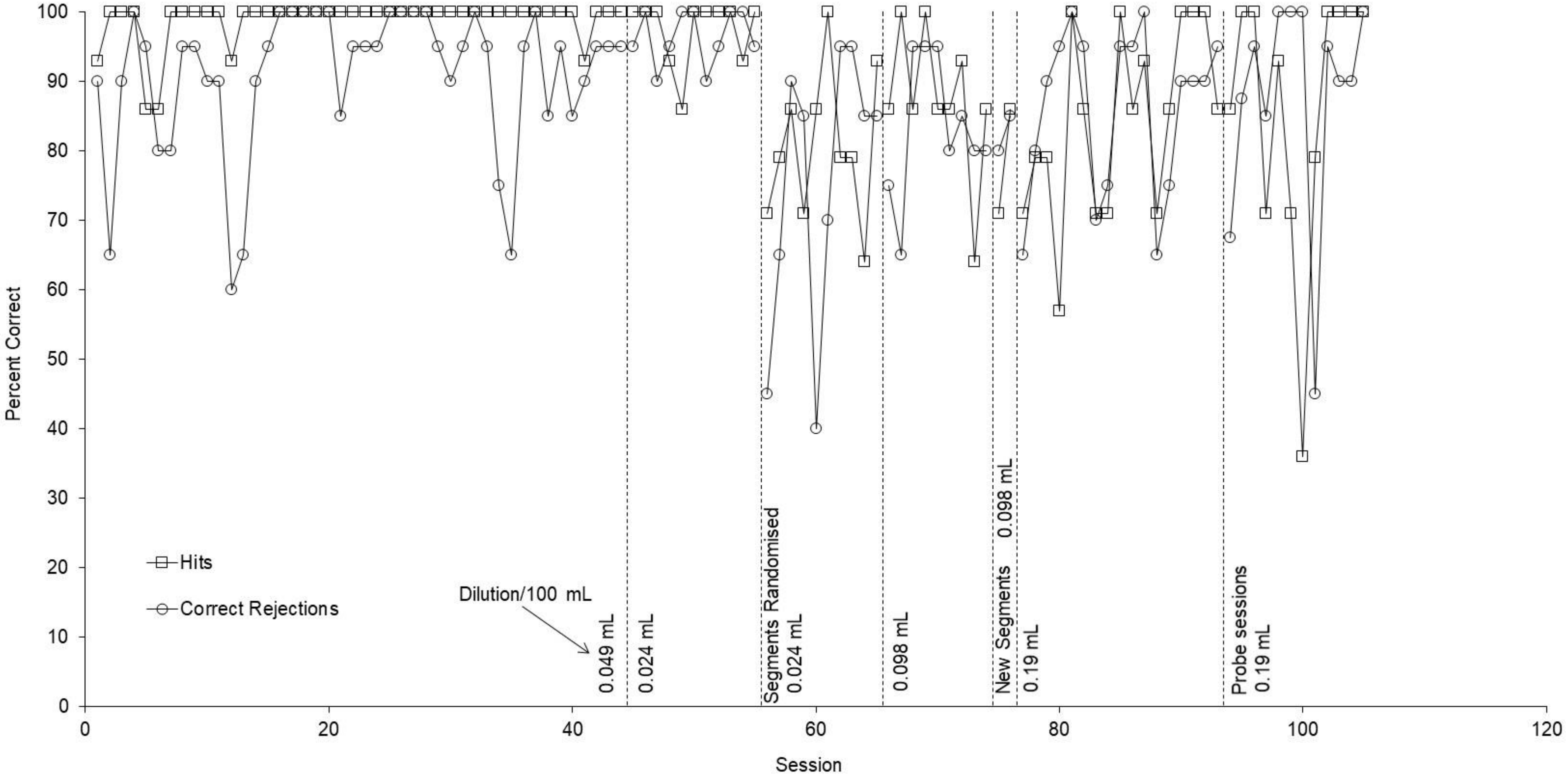

Figure 9. Ruby’s percentage of hits on target (koi carp) samples and the combined correct rejections of non-target (goldfish, no-fish) samples, per session, across conditions.

Multiple-probe sessions

Hits on probe samples were included in analyses only when accuracy rates were at or above 80% for all other samples (koi carp, goldfish and no-fish). This ensured the dogs’ responses to probe samples were accurate indications of their scent-detection thresholds, as high accuracy rates were occurring across sample types. Individual probe session data were collated for each dilution and are shown in Figures 10, 11 and 12 as the average percentage of hits on systematically diluted probe samples.

Subject Mica’s multiple-probe session data are shown in Figure 10. Hits occurred at all koi carp probe sample dilutions (i.e., from 0.098 to 0.00094 mL per 100 mL) with an average hit rate of 60.8% across probes. On average, performance at criterion levels (i.e., at or above 80% accuracy) occurred for only three probes at dilutions of 0.006, 0.003 and 0.00375 mL per 100 mL.

Figure 11 shows subject Luna’s multiple-probe session data. Hits occurred on probe samples at all koi carp concentrations. The bars of Figure 14 show the average percent of hits was quite consistent across probe sample dilutions, with a combined mean of 78.2%. Performance at criterion levels (above 80%) occurred for eight out of 18 probe sample dilutions (0.049, 0.024, 0.012, 0.006, 0.003, 0.0075, 0.00375 and 0.00094 mL per 100 mL).

Subject Ruby was unable to attend the Canine Research Facility for several weeks during the multiple-probe experimental sessions. As shown in Figure 12, her probe sessions were conducted at the beginning (probes 1, 2 and 3) and end (probes, 16, 17 and 18) of the experimental period. Hits occurred at all the koi carp concentrations to which Ruby was exposed. At the highest dilution (0.00094 mL per 100 mL) Ruby’s performance was above criterion levels, with an average hit rate of 87%.

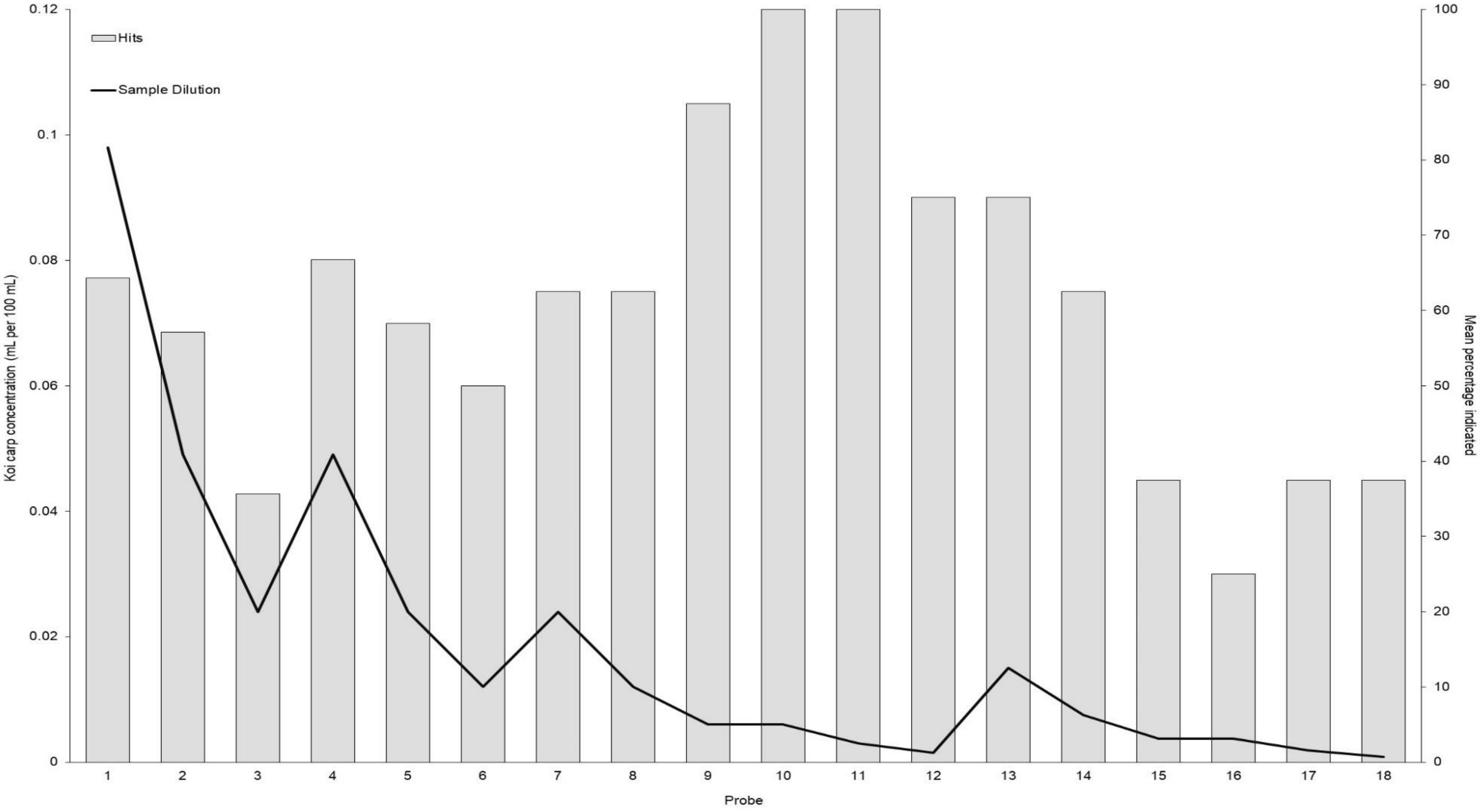

Figure 10. Mica’s mean percentage of hits on systematically diluted koi carp probe samples.

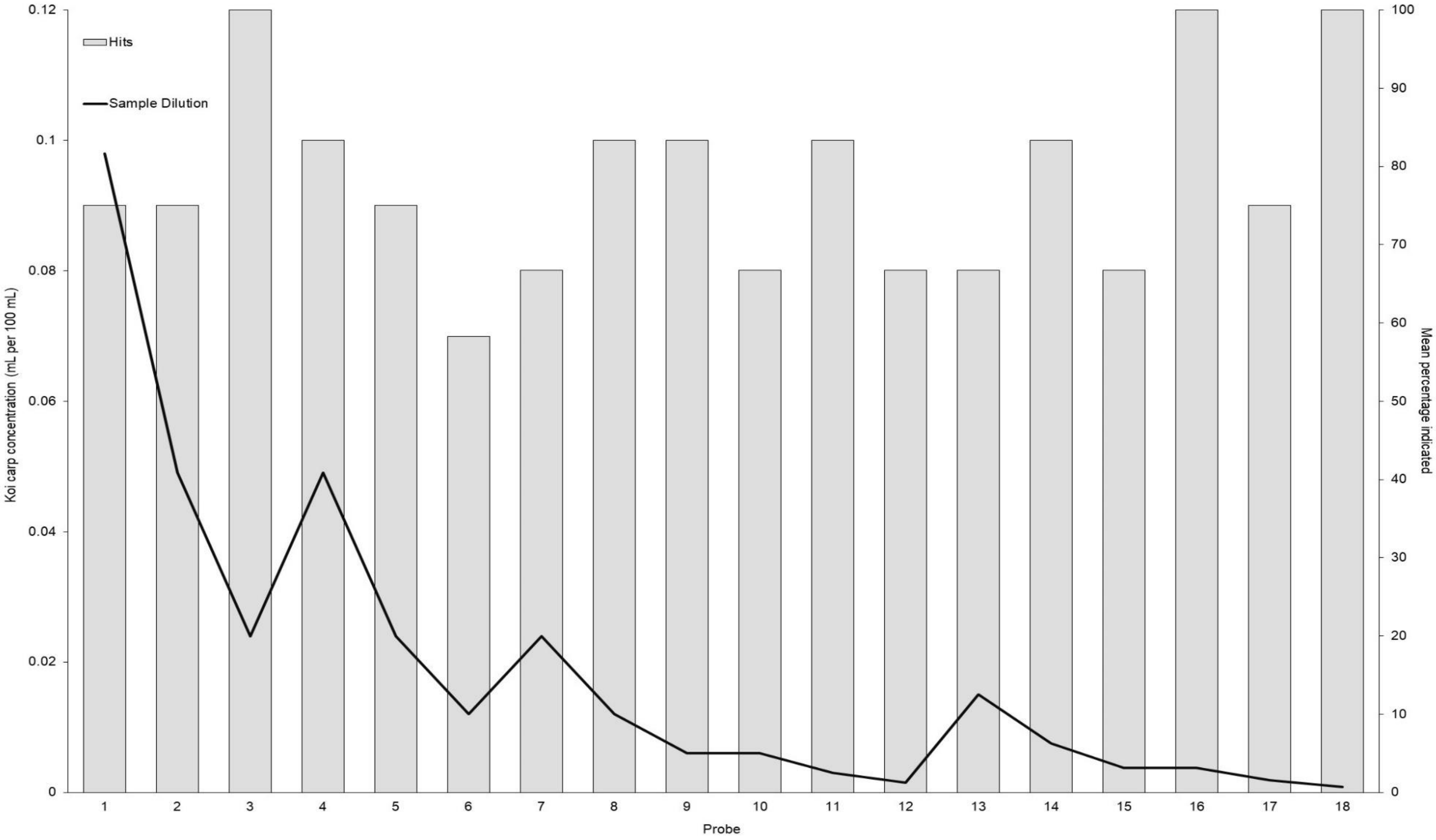

Figure 11. Luna’s mean percentage of hits on systematically diluted koi carp probe samples.

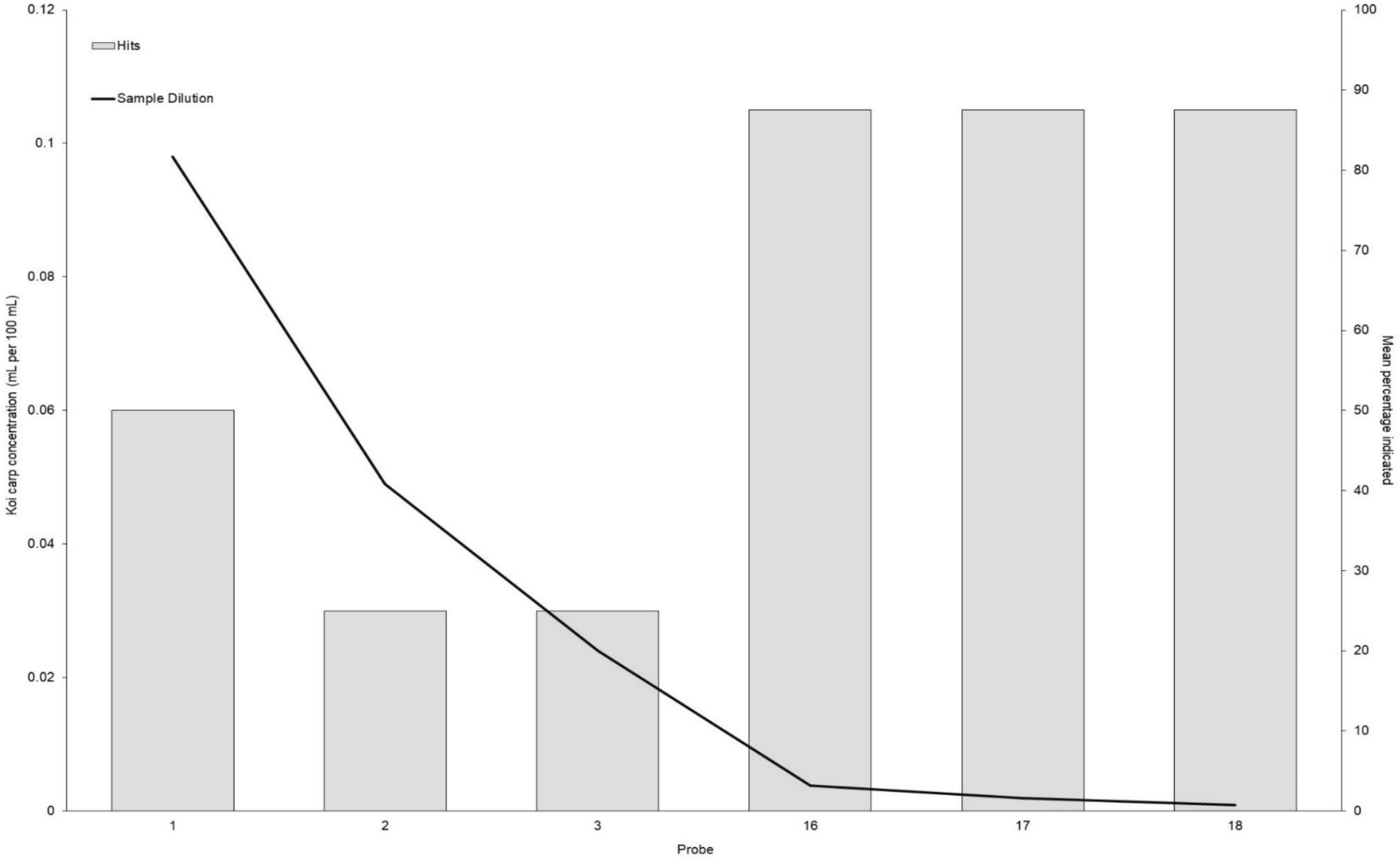

Figure 12. Ruby’s mean percentage of hits on systematically diluted koi carp probe samples.

Session with no samples

In Figures 13 and 14, the percentage of hits on target (koi carp) samples and the correct rejections of non-target (goldfish, no-fish) samples are shown across six sessions of one experimental day. The target (koi carp) and non-target (goldfish) samples were prepared at a dilution of 0.19 mL per 100 mL. In the seventh session, all samples were removed. The segments were arranged in a pre-randomised order on the apparatus and subjects Mica and Luna were run through one session each. The three data points of session seven in each figure show the percentage of hits on segments used for target (koi carp) samples, and the correct rejections of segments used for non-target (goldfish, no-fish) samples, on that experimental day.

Figure 13 shows the performance of subject Mica. Hit rates and correct rejection rates in the first session were below criterion levels (80% accuracy) for all sample types. Accuracy rates then steadily increased, reaching criterion levels by the third session of the experimental day. Hit and correct rejection rates stabilised in the range of 90% to 100% accuracy and converged (100% accurate) by session six. With samples removed, the hit rate on target (koi carp) segments and the correct rejection rates of non-target (goldfish, no-fish) segments were 100% accurate.

Figure 14 shows the performance of subject Luna. On the first session, the correct rejection rates were low (25% to 40% accuracy). In the following sessions, the correct rejection rates steadily increased and were above criterion levels (80% accuracy) by the third session. The hit rate was above 80% accuracy from the first session and remained stable across sessions; hit and correct rejection rates converged by session five (100% accurate). With samples removed, the hit rate on target (koi carp) segments was 100% accurate. The correct rejection rates were 90% accurate for non-target (goldfish) segments and 100% accurate for non-target (no-fish) segments.

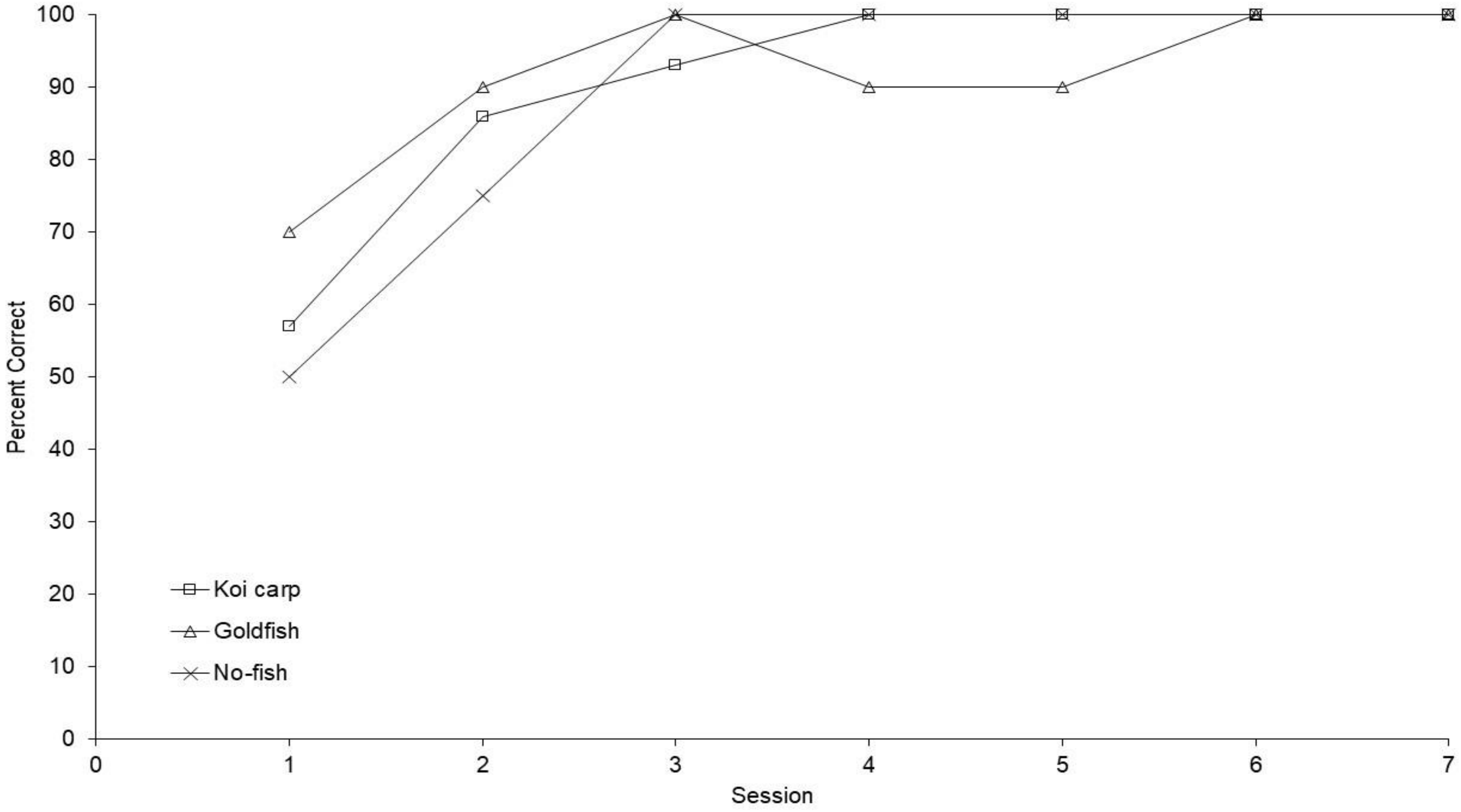

Figure 13. Mica’s percentage of hits and correct rejections of target (koi carp) and non-target (goldfish, no-fish) samples, across six consecutive sessions, of one experimental day. With samples removed, the percentage of hits on segments that had contained target (koi carp) samples and the correct rejections of segments that had contained non-target (goldfish, no-fish) samples, are shown in session seven.

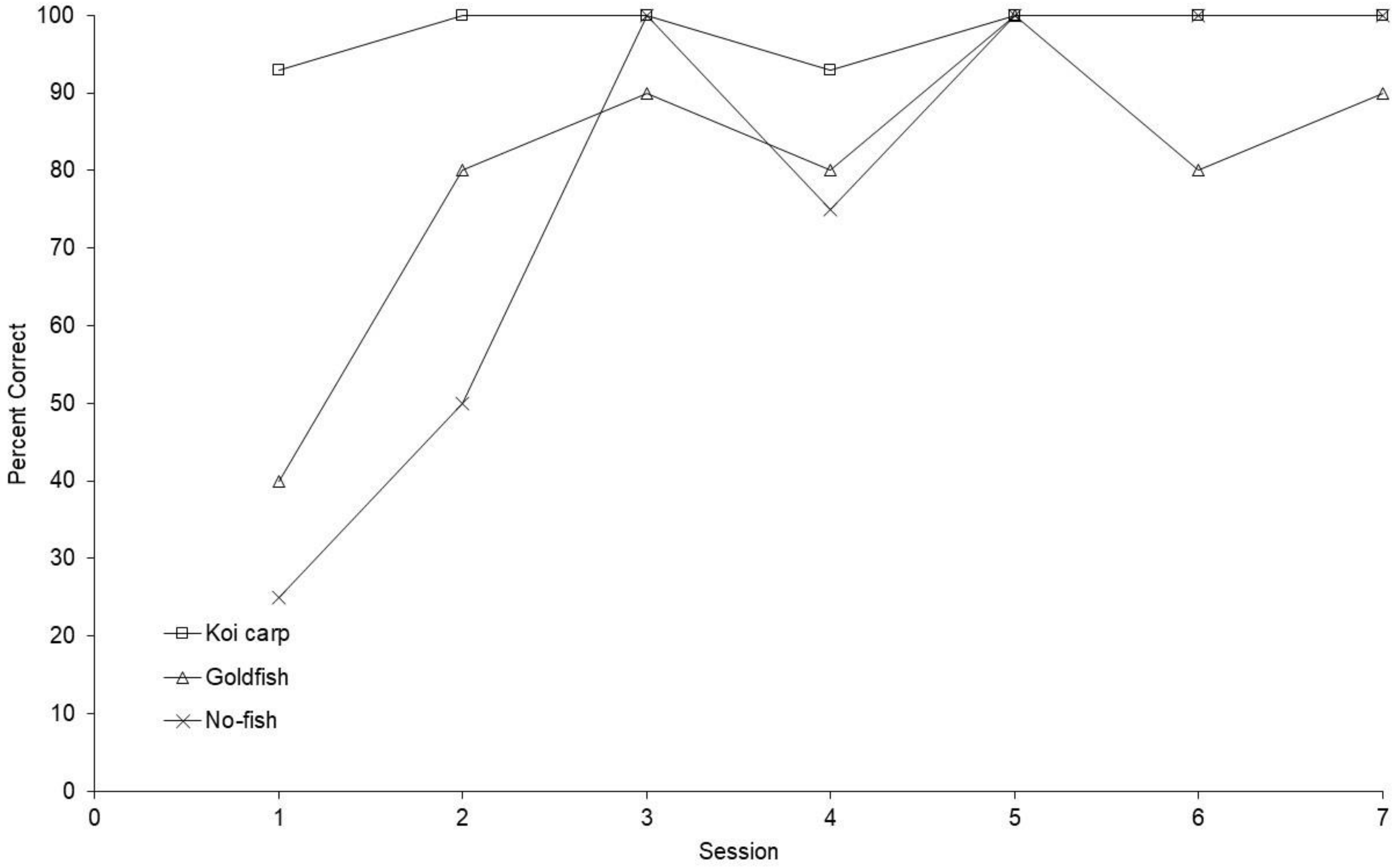

Figure 14. Luna’s percentage of hits and correct rejections of target (koi carp) and non-target (goldfish, no-fish) samples, across six consecutive sessions, of one experimental day. With samples removed, the percentage of hits on segments that had contained target (koi carp) samples and the correct rejections of segments that had contained non-target (goldfish, no-fish) samples are shown in session seven.

First and second trial responding

Sensitivity, specificity, and the overall accuracy of indications on first and second trials, across all experimental sessions, were calculated for each subject. The proportion of positive samples indicated was similar across first and second trials for all dogs, with sensitivities ranging from 91.9% to 100% on first trials and 90.4% to 97.8% on second trials (Table 4). The proportion of negative samples correctly rejected differed between the first and second trials. First trial specificity ranged from 28.1% to 46.6% and second trial specificity was higher for all dogs ranging from 60.7% to 76.4%. Overall accuracy was higher for all the dogs on the second trials.

Table 4

Sensitivity, Specificity and Overall Accuracy of Responses on First Trials and Second Trials of Each Session, per Subject

Subject	Trials	Sensitivity (%)	Specificity (%)	Overall accuracy (%)
Mica	First	91.9	46.6	65.8
	Second	90.4	68.1	77.2
Luna	First	98.5	28.1	58.6
	Second	97.1	60.7	76.4
Ruby	First	100	34.6	57.4
	Second	97.8	76.4	86.1

Chapter 4: Discussion

The present study investigated the scent-detection accuracy of dogs as they learned to discriminate between water samples from aquaria containing koi carp, goldfish or no fish, at systematically diluted concentrations. The reliability of this study's findings was improved through procedural and apparatus modifications following the identification of confounding variables. The three dogs used in this study were found to detect koi carp scent at concentrations equivalent to an effective biomass of 0.4 kg/ha. Accuracy measures were found to increase as sessions progressed within an experimental day. After several sessions, conducting a session with the samples removed did not affect accuracy measures, which suggests residual volatiles may have accumulated in the segments.

Graphical analyses

Previous research using the same subjects, apparatus and phase change criteria (four consecutive sessions at or above 80% accuracy), had reported detection of koi carp scent and discrimination between koi carp, goldfish, and no-fish aquaria water samples at a dilution of 0.098 mL per 100 mL (an effective biomass density of approximately 37.4 kg/ha; Crawford, 2018; Quaife, 2018). During initial experimental sessions in this study, the three dogs met the criterion at a dilution of 0.049 per 100 mL within 30 sessions. This differs from Crawford's (2018) findings in which the dogs did not meet the criterion at the same dilution in 61 (Mica), 72 (Luna), and 52 (Ruby) sessions. However, Crawford (2018) encountered many unfavourable variables (e.g., fish aquaria contamination and dog absences) while conducting experimental sessions, which likely had an effect on the dogs' performance. Quaife (2018) found that, at a 0.098 mL dilution, all three dogs met the criterion within 14 sessions. Similarly, the results from the current study found that once the dogs' performance was stable (i.e., at criterion levels) at the 0.049 mL dilution, two of the dogs met the criterion within 10 sessions at a much higher dilution of 0.024 mL per 100 mL (an effective biomass density of approximately 9.2 kg/ha). As it was expected that the dogs' performance would take longer to reach criterion accuracy levels, this finding raised scepticism of the study's internal validity and led to the implementation of a control test.

Control test

Both DeShon et al. (2016) and Railton (2011) conducted sessions with no target stimuli to assess control of responding by extraneous stimuli. DeShon et al. (2016) tested four dogs trained in the detection of quagga mussel veliger scent, by conducting a session with no target samples present; instead, all samples contained control water. It was found that none of the dogs alerted to any sample presented in the control test session. Similarly, Railton (2011) tested hens trained in a discrimination task, by conducting sessions with the discriminative stimuli removed from the apparatus. In this case, for most of the hens, accuracy did not change much from when sessions were conducted with the stimuli, indicating that some of the hens were obtaining cues from extraneous variables.

Unlike DeShon et al. (2016), and similar to Railton (2011), this study's control test, conducted with Mica and Luna, indicated that extraneous variables were most likely influencing detection rates. It was expected that the 17 control (no-fish) samples would be correctly rejected by both dogs. However, the findings showed this did not occur because Mica and Luna indicated on segments that were used exclusively for koi carp samples, even though these segments did not currently contain sampled koi carp aquarium water. This was illustrated by the pattern of responses with many positive indications occurring on control (no-fish) samples in target (koi carp) segments and very few on control (no-fish) samples in non-target (goldfish, no-fish) segments. Mica’s positive indications on target (koi carp) segments were observed to decrease in the second round (trials 18 to 34), likely due to no reinforcement being provided for these indications in the first round. Luna lay down in the experimental room after only eight trials. Both these behaviours have been observed with dogs when conducting extinction procedures (Bentosela, 2008). This suggests that the opportunity to emit a hit response was available, even though all samples contained control (no-fish) water. Of course, false alarms could occur, but considering a larger response cost was associated with a positive indication (hit) than a correct rejection, false alarms would be expected to occur infrequently, and randomly with regard to segment type. One possible explanation is that the segment cleaning procedure may not have been sufficient at removing all traces of fish-related scent from the segments. Therefore, segments that had been used exclusively for target (koi carp) samples, may still have contained some koi carp fish-related VOCs which could be detected by the dogs. Other explanations could be that the dogs were responding to other features of the segments such as variation in the materials, or some other residual material.

After controlling for extraneous cues, Railton (2011) found that for all but one hen, accuracies decreased to levels predicted by chance. In this study, modifications to the cleaning procedure and the unsystematic placement of segments (i.e., segment randomisation) were implemented to control for extraneous cues. Resuming sessions at a 0.024 mL per 100 mL dilution showed the dogs' accuracy measures decreased for all sample types. It is important to consider that conducting a session with no target (koi carp) samples present could lead to a decline in performance in subsequent sessions (Porritt et al. 2015). However, no control test session was conducted with Ruby, yet the same decrement in performance was observed.

The dogs' performance did not meet the criterion after increasing the concentration to 0.098 mL per 100 mL. This is understandable, as a form of retraining had to occur without the extraneous cues. Despite improvements to the segment cleaning procedure and the randomisation of segments, extraneous cues associated with the segments (i.e., fish-related VOCs or other segment features) may still have been detected by the dogs. To control for possible extraneous cues, the segments were replaced with 17 new segments. The dogs then met the criterion at a dilution of 0.19 mL per 100 mL (an effective biomass density of approximately 72.5 kg/ha). Most likely, if more sessions had been conducted at a dilution of 0.098 mL with the new segments, the dogs would have eventually met the criterion, supporting the findings of previous studies (Crawford, 2018; Quaife, 2018).

Hits and combined correct rejections

A response bias was reported by Quaife (2018) with the three dogs (Mica, Luna, Ruby) overall displaying higher hit rates than combined correct rejection rates when discriminating between target (koi carp) and non-target (goldfish, no-fish) samples. Similarly, in this study, the hit rates of Mica and Luna were found to be higher than the combined correct rejection rates across all experimental phases.

Ruby’s hit rate was initially higher than her combined correct rejection rate, however this trend reversed when new segments were introduced to experimental sessions.

Ellis, Mulder, Valverde, Poling, and Edwards (2017) reported overall higher measures of reliability in the detection of positive tuberculosis (TB) sputum samples by African giant pouched rats (*Cricetomys ansorgei*) compared to negative sputum samples. It was suggested that some of this disparity could be related to the procedural design of the study, in that only indications on positive TB samples were reinforced. This could apply to the hit and combined correct rejection rate findings in the current study as a go/no-go procedure was used where only hits (positive indications on target samples) were reinforced. However, more research would need to be conducted with a larger number of dogs to ascertain if there was any effect of the research design on the dogs’ responses.

It is not uncommon for dogs used in the same research, even when trained in the same manner, to display differences in detection rates (Goodwin et al. 2010; Lin et al., 2011). For example, the findings of the current study showed that during some experimental phases the difference between hit and correct rejection rates reached over thirty percent with Luna, but never exceeded eight percent with Ruby. For Ruby, this shows that even though her combined correct rejection rates were higher than her hit rates in the last three experimental phases, the difference between her hit and correct rejection rates were minimal. Similarly, Ellis et al. (2017) found out of 22 African giant pouched rats, one rat was equally reliable at indicating on both positive and negative sputum samples. These findings show that there can be quite a bit of variability in performance between individual animals used in research, and that with a larger sample size, patterns of responses may become clearer.

Multiple-probe sessions

The rationale for the multiple-probe experimental design was based on the following considerations. It is likely that if indications (hits) on probe samples were reinforced, then the probability of the dogs indicating on probe samples of the same concentration again would increase in subsequent sessions. Alternatively, by not providing reinforcement for indications (hits) on probe samples, there was the possibility of the positive indication behaviour extinguishing as the dogs were not used to the intermittent reinforcement of target samples. In both cases (providing reinforcement or not), the probability of the dogs indicating on a probe sample would probably change. From an operational perspective, not reinforcing indications (hits) on probe samples more closely resembled the application of dog scent detection in a real-life scenario, as the presence of the target scent in a sample taken from a waterbody (e.g., a lake) would be unknown. Therefore, any positive indications on the sample would not be reinforced even though the sample may contain the scent of koi carp fish.

The possibility predicted by not reinforcing indications (hits) on probe samples did not eventuate, with the findings showing that the dogs would continue to ‘work’ under intermittent reinforcement. Similar findings have been reported by Baer, Peterson, and Sherman (1967) who conducted probe trials with children trained to imitate another person’s behaviour. Initially, behaviour was shaped by providing food and praise for the imitation of a response. Once the modelled behaviours were imitated reliably, probe trials, which were not reinforced, were included randomly among reinforced trials. Imitative responses continued to occur in both reinforced and unreinforced trials with similar rates of imitative behaviour in both trial types. This is an important finding for the current research as it provides evidence for the utility of dogs in the detection of fish-related VOCs from water samples in a real-life operational scenario. That is, the dogs will continue to indicate at lower concentrations than the training concentration and without continuous reinforcement.

The dogs were able to detect koi carp scent at all of the concentrations tested (from 0.098 mL/100 mL to 0.00094 mL/100 mL), equivalent to an effective biomass range of between 72.5 and 0.4 kg/ha. In laboratory prepared water samples containing the diluted compounds of either 2-methy-lisoborneol or geosmin in 1.5 mL microcentrifuge tubes, Shelby, Schrader, Tucker, Klesius and Myers (2004) reported scent detection of the samples by three dogs, at very low concentrations of 1 mg/L^{-1} and 1 $\mu g/L^{-1}$, and found lowering the concentration to 10 ng/L^{-1} was followed by a decrease in performance for all the dogs. In the current study, no clear relationship was observed between dilution and indication rate for any of the dogs. All three dogs indicated with higher accuracy on some of the lowest concentrations tested compared to higher concentrations, even though every probe session and any associated variables were held constant by testing three probe samples, each of a different concentration, per experimental day. A thorough investigation found no evidence of any systematic difference corresponding with the preparation or presentation of the probe (koi carp) samples which could have been responsible for these outcomes. These findings show that the three dogs used in this study have exceptional olfactory acuity for the fish-related VOCs of adult koi carp, and suggests that they may be able to detect koi carp scent at even lower concentrations.

Session with no samples

The graphs of the *Session with no samples* show a typical pattern of responses observed during experimental sessions within an experimental day. This repeating pattern of low first session accuracy followed by increasing accuracy as sessions progressed within an experimental day occurred in sessions throughout the experimental phases, but was more obvious during the multiple-probe sessions. This was likely because the identified extraneous cues were controlled for and the response pattern had become more stable compared to earlier experimental phases following the control test session. The two no sample sessions were conducted to further explore this pattern of increasing accuracy.

Studies have found that dogs' scent detection accuracy tends to increase over time with repeated exposure to the target (Cablk & Heaton, 2006; Shelby et al. 2004). However, in the current study, high rates of accuracy achieved by the end of an experimental day did not carry over to the next experimental day; instead the pattern (i.e., low first session accuracy incrementally increasing with each session) was repeated each experimental day. Interestingly, the findings of the *Session with no samples* showed after conducting six sessions with the segments and sample types held constant, conducting a further session with no samples, did not affect the dogs' accuracies which remained very high and consistent with usual results.

From these findings it could be suggested that the pattern of responses might be related to the accumulation of the fish-related VOCs from each sample into its respective segment. Physical properties of VOCs such as low to medium water solubility and low molecular weight means they evaporate readily at ambient temperature (Bennett & Inamdar, 2015; Fink, 2007). Once the apparatus was set up, at least 15 minutes elapsed before a session was started. Similar to Concha et al. (2014), this was done to allow the target and non-target scents to permeate the headspace of the segments as the water samples acclimatized to the experimental room temperature. However, it is possible that only a small percentage (only those VOCs at the surface of the water samples) of the fish-related VOCs in the samples had evaporated after 15 minutes, with more VOCs evaporating with time and temperature changes across the experimental day. For example, the temperature within each segment was likely temporarily increased through the inspired and expired air of the dogs' sniffing behaviour, enhancing the evaporation of the fish-related VOCs (Craven et al., 2009). Temperature is one factor that would increase the evaporation of fish-related VOCs, hence the dogs' detection accuracy would probably increase with each successive session within an experimental day as more VOCs evaporated from the samples.

However, after each set of sessions, the samples and segments were removed from the apparatus so that they could be re-randomized, which means the air inside the segments would have dispersed into the wider environment. Therefore, it could be argued that as the fish-related VOCs evaporated from the samples into the headspace of the segment, some of the evaporated VOCs remained on the inside of the metal segments. This is supported by the control test session which showed fish-related scent had accumulated in the segments, and by Quaife (2018) who found contamination occurred if segments were switched between sample types each session set. When no samples were present in the

Session with no samples, Mica and Luna achieved hit and correct rejection accuracy rates of between 90% and 100%. This further supports this claim, as it appears enough fish-related VOCs were present in the segments for the dogs to detect the target scent.

Increasing accuracy could also be related to cues from the dogs themselves. For example, in the current study, the dogs had to leave their nose in the port for a longer amount of time when indicating positively compared to negatively. Research has found that dogs sniff for a longer amount of time when making a positive indication compared to a negative indication (Concha et al. 2014), which could provide cues in the form of dog scent to other dogs in subsequent sessions (Lit et al., 2011). Studies have controlled for this possibility by cleaning the sample containers between each dog's session (DeShon et al., 2016) and by allocating separate sample containers for each dog used in the research (Alexander et al., 2015). Dog scent cues may have contributed to the dogs' increasing accuracy with each successive session in an experimental day. However, the first dog's first evaluation of the samples (trials 1 to 17) would not have been contaminated with any dog scent, yet correct indications on some target (koi carp) and non-target (goldfish and no-fish) samples still occurred.

In summary, it is difficult to tease apart these factors as they are related. For example, if more sniffing is occurring with positive indications, then more VOCs may evaporate, and with repeated positive indications more dog scent may be present on these segments, together influencing detection accuracy. It is plausible that both VOC dispersal and dog scent cues were contributing to the dogs' increasing accuracy rates as sessions progressed within an experimental day, to some extent.

First and second trial responding

Comparing first trial and second trial accuracy measures (hits and correct rejections) indicated that sensitivity was similar on first and second trials whereas specificity increased for all dogs on second trials. This is interesting as samples were randomized each session and there was always a slightly greater likelihood of a non-target sample being in position 1 on the apparatus due to the higher number of non-target samples (10 non-target, 7 target samples). Indications by dogs based on sample position, regardless of target presence, has been observed in other research (Angle et al., 2015; Browne et al., 2015; DeShon et al., 2016; Goodwin et al., 2010). An approach used by Goodwin et al., (2010) and Angle et al., (2015) to combat this tendency of some dogs was to include trials with no targets present, so that the dogs learnt that a target sample would not always be available in a session. This could be included in the training protocol of dogs utilised in koi carp scent detection research, although both those studies used a much smaller number of samples (less than 9) and therefore fewer trials per session, compared to the current study (17 samples, 34 trials). From an operational perspective, it is important for the dogs to respond to each sample equally, without a bias towards a sample position in the apparatus. Further research could consider conducting some sessions consisting of fewer trials with no target samples present, to ascertain whether this would be an effective way to manage the dogs' first trial positive indication tendencies.

Implications

This research has demonstrated that scent-detection dogs can detect the scent of koi carp from water samples at effective biomass densities of between 0.4 and 72.5 kg/ha. This range of detection implies that dogs have utility in contributing to the detection of koi carp and, in particular, of suspected recent incursions. This is very important as the negative environmental effects of carp are cumulative, increasing as populations grow (Badiou & Goldsborough, 2010; Bajer et al. 2009). Early detection can inform fisheries management of where the implementation of control measures is necessary, preventing progressive environmental degradation.

This research has also demonstrated the applicability of scent-detection dogs for operational scenarios. The multiple-probe experimental sessions showed the dogs will continue to assess samples when intermittently reinforced, and when these samples contain different concentrations of target scent. This type of reinforcement with samples of variable concentrations resembles a real-life operational situation. Further investigation by researchers into how accurate responses by the scent-detection dogs could be achieved in one experimental session of sample presentation instead of multiple experimental sessions with the same samples would extend this applicability.

The significance of this research for future studies in this area applies to the research methods. This research has demonstrated the importance of controlling for confounding variables. Researchers need to be vigilant of unintended sources of control, ensuring that the observed detection rates are accurate of dogs' ability to detect the target, uninfluenced by other factors. Also, understanding how quickly the target scent is dispersed from samples and which factors might influence this dispersal is important. Further research could look at modifying the current sample presentation method, possibly by reducing the sample volume (Shelby et al. 2004) or testing different mediums of sample presentation in which a known concentration is dispersed at a known rate (Mendel et al., 2018; Simon et al., 2017). To reduce bias, researchers could look at implementing blank trials to control for automatic first session positive indications (Angle et al., 2015; Goodwin et al., 2010), and possibly manipulating response costs to even out hit and correct rejection rates (Tashkoff, 2017). Having a solid research method will be the basis for then progressing to real-world applications of scent-detection dogs for koi carp detection.

Limitations

Although many dogs were recruited for this study, most did not pass the selection criteria. Therefore, the generalizability of this study's findings is limited as they apply directly only to dogs which have passed similar screening procedures.

Following the control test, control measures were put in place to ensure extraneous cues and contamination were not occurring. However, a potential methodological issue which was not addressed was the placement of stickers on only some sample types. To make identification of sample types straightforward, small white stickers were placed on target (koi carp), non-target (goldfish) and probe (koi carp) samples, but not on non-target (no-fish) samples. Considering the dogs' olfactory sensitivity, even a slight odour from the sticker, such as from the sticker adhesive could have provided an additional source of scent associated with samples other than the non-target (no-fish) samples. It is unlikely that this occurred as the dogs were able to discriminate between koi carp and goldfish samples even though they both had stickers placed on the outside of the sample jars.

Another methodological issue was the refreshment of samples. Initially, after several experimental sessions had been conducted in the morning, new samples were prepared around midday so that the evaporated fish-related VOCs were replenished. Refreshment of samples was eventually discontinued (at the beginning of the multiple-probe sessions) as an increase in accuracy was observed after samples were refreshed. This made sense once the *Session with no samples* was conducted, showing that the fish-related VOCs were likely accumulating on the inside of the segments. Therefore, the procedure of refreshing the samples would have actually increased the concentration of the fish-related VOCs, giving accuracy measures at a higher concentration than the original concentration. This may have inflated some of the accuracy measures recorded.

Future research

This research has provided support for the utility of dogs for koi carp detection. Specifically, this study has investigated dog scent-detection accuracy with a range of sample concentrations, showing that dogs can detect the scent of koi carp from sampled aquaria water at biomass estimates that could be found in various aquatic environments. With proper control of variables, future research could look at replicating the multiple-probe design experiment to ascertain a detection threshold. Further development of this experiment could involve interspersing laboratory samples with water samples taken from natural (i.e., field) waterbodies containing a known approximate koi carp biomass. Researchers could then evaluate the impact of additional environmental odours (e.g., aquatic plants, other organisms, and other organic matter odours) on dog scent-detection accuracy, and therefore provide further evidence regarding their operational applicability.

Conclusion

Koi carp are a pervasive, environmentally harmful species. Ongoing monitoring, necessary for the containment of koi carp, requires better detection methods. This study has shown that dogs can detect koi carp scent at environmentally relevant concentrations and perform under operationally significant scenarios. This study has contributed to the development and evaluation of methods for research involving scent-detection dogs for koi carp detection through the identification of confounding variables and consideration of how the research methods can be improved to enhance experimental control. Based on these findings, future research should provide more accurate and reliable data for the development of practical measures involving scent detection to control koi carp.

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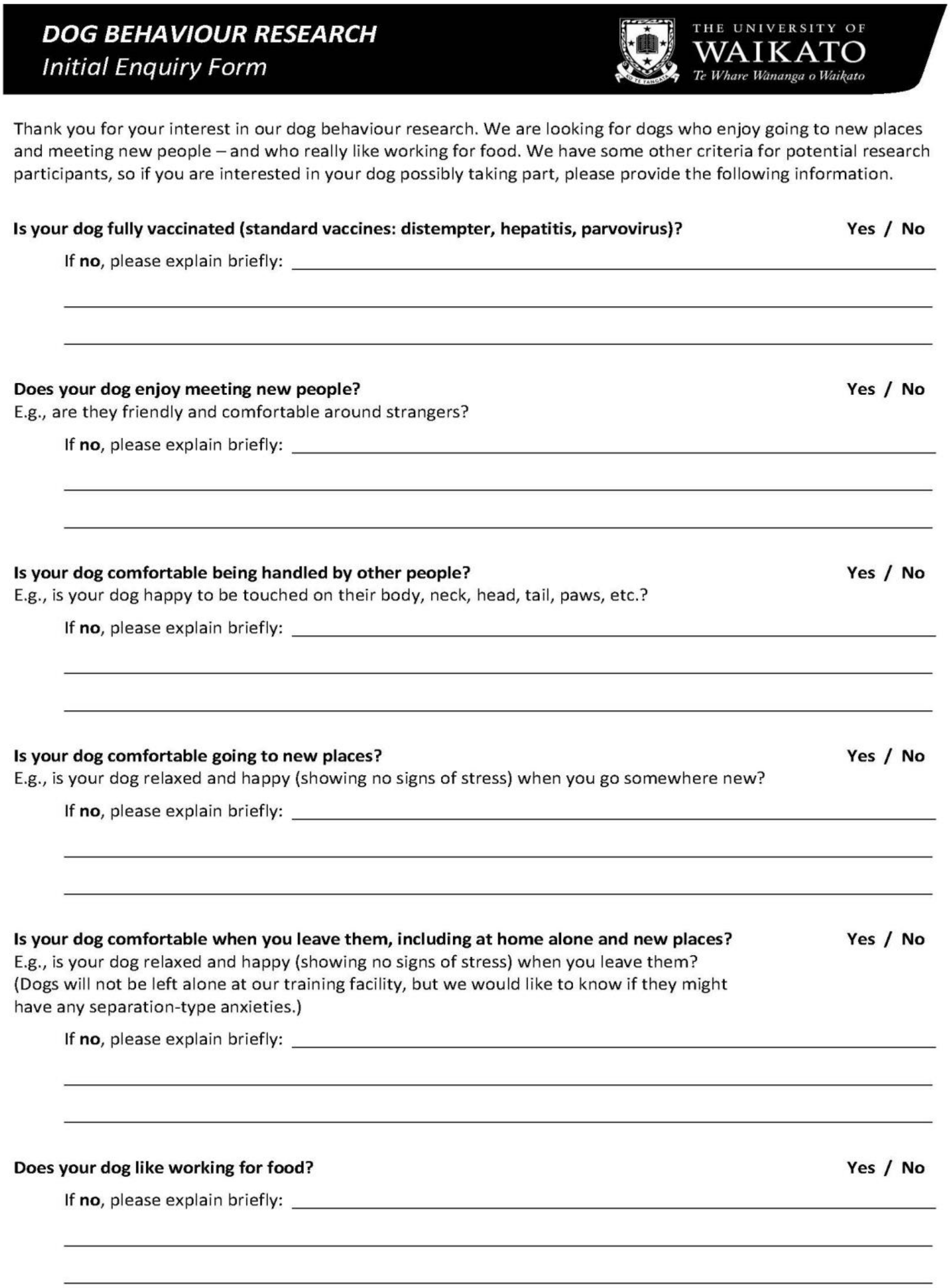
Appendix A



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 will not be left alone at our training facility, but 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 aggrevated 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 Univeristy of Waikato main campus)? **Yes / No**

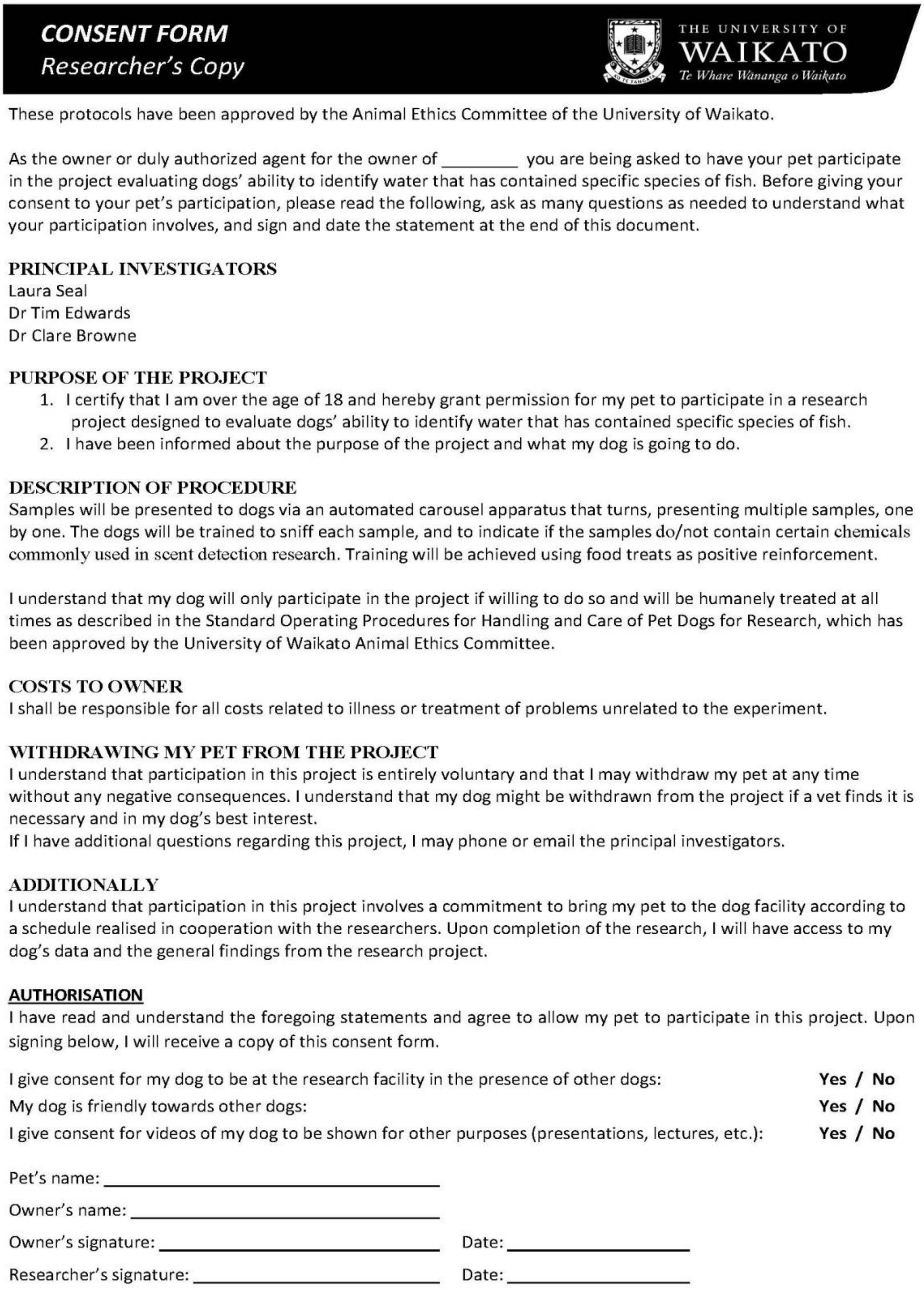
Please indicate which times are more convenient: ______________________________

____

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:

Appendix B



CONSENT FORM
Researcher's Copy

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

These protocols have been approved by the Animal Ethics Committee of the University of Waikato.

As the owner or duly authorized agent for the owner of ________ you are being asked to have your pet participate in the project evaluating dogs' ability to identify water that has contained specific species of fish. Before giving your consent to your pet's participation, please read the following, ask as many questions as needed to understand what your participation involves, and sign and date the statement at the end of this document.

PRINCIPAL INVESTIGATORS
Laura Seal
Dr Tim Edwards
Dr Clare Browne

PURPOSE OF THE PROJECT

1. I certify that I am over the age of 18 and hereby grant permission for my pet to participate in a research project designed to evaluate dogs' ability to identify water that has contained specific species of fish.
2. I have been informed about the purpose of the project and what my dog is going to do.

DESCRIPTION OF PROCEDURE
Samples will be presented to dogs via an automated carousel apparatus that turns, presenting multiple samples, one by one. The dogs will be trained to sniff each sample, and to indicate if the samples do/not contain certain chemicals commonly used in scent detection research. Training will be achieved using food treats as positive reinforcement.

I understand that my dog will only participate in the project if willing to do so and will be humanely treated at all times as described in the Standard Operating Procedures for Handling and Care of Pet Dogs for Research, which has been approved by the University of Waikato Animal Ethics Committee.

COSTS TO OWNER
I shall be responsible for all costs related to illness or treatment of problems unrelated to the experiment.

WITHDRAWING MY PET FROM THE PROJECT
I understand that participation in this project is entirely voluntary and that I may withdraw my pet at any time without any negative consequences. I understand that my dog might be withdrawn from the project if a vet finds it is necessary and in my dog's best interest.
If I have additional questions regarding this project, I may phone or email the principal investigators.

ADDITIONALLY
I understand that participation in this project involves a commitment to bring my pet to the dog facility according to a schedule realised in cooperation with the researchers. Upon completion of the research, I will have access to my dog's data and the general findings from the research project.

AUTHORISATION
I have read and understand the foregoing statements and agree to allow my pet to participate in this project. Upon signing below, I will receive a copy of this consent form.

I give consent for my dog to be at the research facility in the presence of other dogs: **Yes / No**
My dog is friendly towards other dogs: **Yes / No**
I give consent for videos of my dog to be shown for other purposes (presentations, lectures, etc.): **Yes / No**

Pet's name: ______________________________
Owner's name: ____________________________
Owner's signature: __________________________ Date: ________________
Researcher's signature: _______________________ Date: ________________

Appendix C

Standard Operating Procedure: Water Sample Preparation and Collection

Guidelines for Water Sample Preparation and Collection

1. Purpose

This standard operating procedure (SOP) provides guidelines and standardised procedures to be adopted during the preparation and collection of water samples from the Aquatic Research Centre, located at FC2-G on the University of Waikato campus. Only those with prior induction training are authorised to collect samples.

2. Sample Preparation

2.1. Covered shoes must be worn on the premises at all times.

2.2. Place all personal belongings in office (Room G.01 on facility map).

2.3. Put on a pair of disposable gloves.

2.4. Adjust the water flow of the control (no-fish) aquarium by turning the blue lever above the aquarium to the horizontal ‘off’ position.

2.5. Remove the plug pipe from the plug hole and lean it against the wall, in a corner of the control aquarium.

2.6. Retrieve the scouring pad from the plastic container underneath the control aquarium.

2.7. Use the scouring pad to gently scrub all inside surfaces of the aquarium.

2.8. Return the scouring pad to the plastic container.

2.9. Adjust the blue lever so that a steady flow of water is running and rinse all aquarium surfaces.

2.10. Place the plug back into the plug hole.

2.11. Fill the aquarium with water up to the first row of holes on the plug pipe.

2.12. Adjust the blue lever so that the water is flowing at a slow steady rate.

2.13. Check that the aeration bubbler is working and that it is under the surface of the water.
2.14. Remove and dispose of gloves.
2.15. Put on a new pair of disposable gloves.
2.16. Remove mesh net from the goldfish aquarium and place on the stainless steel bench.
2.17. Adjust the water flow by turning the blue lever above the aquarium to the horizontal ‘off’ position.
2.18. Remove the plug pipe from the plug hole and lean it against a wall, in a corner of the aquarium.
2.19. Drain the water from the aquarium until the goldfish are covered by water at a minimum level and put the plug pipe back into the plug hole.
2.20. Retrieve the scouring pad from the plastic container underneath the goldfish aquarium.
2.21. Repeat step 2.7 and avoid contact with the fish.
2.22. Return the scouring pad to the plastic container underneath the aquarium.
2.23. Adjust the blue lever so that a steady flow of water is running and rinse all aquarium surfaces.
2.24. Remove the plug pipe from the plug hole and drain the water until the water is running clear. Make sure the fish are always covered by water.
2.25. Replace the plug pipe and fill the aquarium to the first row of holes on the plug pipe.
2.26. Turn the water flow off by adjusting the blue lever to the horizontal ‘off’ position.
2.27. Check that the aeration bubbler is working and that it is under the surface of the water.
2.28. Replace the mesh net over the goldfish aquarium.
2.29. Dispose of gloves.
2.30. Put on a new pair of disposable gloves.
2.31. Remove mesh net from koi carp aquarium and place on top of the freezer.

2.32. Adjust the water flow by turning the blue lever above the koi carp aquarium to the horizontal ‘off’ position.

2.33. Remove the plug pipe from the plug hole and lean it against a wall, in a corner of the aquarium.

2.34. Drain water from the aquarium until the koi carp fish is covered by water at a minimum level and put plug pipe back into the plug hole.

2.35. Retrieve the scouring pad from the plastic container underneath the koi carp aquarium.

2.36. Repeat step 2.7 and avoid contact with the fish.

2.37. Return the scouring pad to the plastic container underneath the aquarium.

2.38. Adjust the blue lever so that a steady flow of water is running and rinse all aquarium surfaces.

2.39. Remove plug pipe from plug hole and drain the water until water is running clear. Make sure the koi carp fish is always covered by water.

2.40. Replace aquarium plug pipe and fill aquarium up to the blue line marked on the aquarium walls.

2.41. Turn the water flow off by adjusting the blue lever to the horizontal ‘off’ position.

2.42. Check that the aeration bubbler is working and is under the surface of the water.

2.43. Replace mesh net on koi carp aquarium.

2.44. Dispose of gloves.

2.45. Lock the door on exiting the building.

2.46. Repeat cleaning procedure after sample collection on Thursday mornings.

3. Sample Collection

3.1. Covered shoes must be worn on the premises at all times.

3.2. Place all personal belongings in office (Room G.01 on facility map).

3.3. Place control (no-fish) sample bottles (in wire carrier) on the floor, close to the control (no-fish) aquarium.

3.4. Place the backpack containing the positive (koi carp) and negative (goldfish) sample bottles on the floor, next to the freezer.

3.5. Put disposable gloves on.

3.6. Cover a section of the sink bench with paper towels. Place 6 paper towels on top.

3.7. Dispose of gloves.

3.8. Put on a new set of gloves.

3.9. Retrieve the (-) beaker from the (-) plastic bag in the storage container under the control (no-fish) aquarium.

3.10. Using the (-) beaker, fill each control (no-fish) sample bottle, taking care not to touch the water with gloved hands or the rim of either sample bottle with the beaker. Replace lids on sample bottles.

3.11. Place the filled sample bottles back into the wire carrier.

3.12. Take the (-) beaker to the sink.

3.13. Turn on the hot water tap. Pour a small amount of dishwashing liquid onto the beaker and wash the beaker under the running water. Turn tap off.

3.14. Using one of the paper towels, dry the beaker.

3.15. Put the clean beaker back into the (-) plastic bag and return to the storage container under the control (no-fish) aquarium.

3.16. Dispose of gloves.

3.17. Put on a new pair of gloves.

3.18. Remove the goldfish aquarium mesh net and place it on the stainless-steel bench.

3.19. Retrieve the (-G) goldfish beaker from the (-G) plastic bag in the storage container.

3.20. Using the (-G) goldfish beaker, fill up the goldfish sample bottle to approximately 400 ml, taking care not to touch the water with gloved hands or the rim of the sample bottle with the beaker. Replace lid on sample bottle.

3.21. Place the goldfish sample bottle into the goldfish Glad® Snap® Lock bag and place in the backpack.

3.22. Take the (-G) goldfish beaker to the sink and repeat steps 3.13 to 3.14.

3.23. Put the clean beaker back into the (-G) plastic bag and return to the storage container under the control (no-fish) aquarium.

3.24. Replace the mesh net over the aquarium.

3.25. Repeat steps 3.16 and 3.17.

3.26. Remove the koi carp aquarium mesh net and place on storage containers opposite the koi carp aquarium.

3.27. Retrieve the (+) koi carp beaker from the (+) plastic bag in the storage container.

3.28. Using the (+) beaker, fill up the positive (koi carp) sample bottle with approximately 400 ml of koi carp aquarium water, taking care not to touch the water with gloved hands or the rim of the sample bottle with the beaker. Replace lid on sample bottle.

3.29. Place the koi carp sample bottle into the koi carp Glad® Snap® Lock bag and place in the backpack.

3.30. Take the (+) koi carp beaker to the sink and repeat steps 3.13 and 3.14.

3.31. Put the clean beaker back into the (+) plastic bag and return to the storage container under the control (no-fish) aquarium.

3.32. Replace the mesh net over the koi carp aquarium.

3.33. Dispose of gloves.

3.34. Turn extreme left light from ‘Manual’ to ‘Automatic’.

3.35. Place rubbish in bin on exiting the building.