Quantifying Links Between Group Size, Temperature Preference, and Behavioural Fever in a Gregarious Cyprinid Using a New Approach to Deconvolve Individual Contributions to Shoaling

by

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Abstract

Shoaling and schooling play crucial roles in survival for over half of all vertebrate fish taxa. Social behaviour in fish reduces stress, increases collective vigilance towards predators, increases foraging productivity, and enhances communication between those electing to remain with the group. Some taxa are obligate schoolers, while others group up gregariously, occasionally shoaling in loose social groups.

Thermal stress caused by anthropogenic climate change may disrupt social behaviours by creating pressure on fish to alter their temperature preference, a behaviourally regulated range of temperature that optimizes physiological rates, fertility, growth, and maturity. Temperature preference also has a role in behavioural fever, where upon infection fish locate zones 2-4°C hotter to enhance innate defense pathways, similarly to metabolically induced fever in endotherms. This thesis presents an experimental approach to quantify the links between shoaling, thermal preference, and behavioural fever by combining a novel thermal enclosure and machine-learning multitracking protocol.

It is a challenge to properly assess shoaling behaviour and thermal preference within experimental aquaria. Previous studies have been limited in simulating realistic environmental gradients and avoiding systematic rheotactic and thigmotactic biases. This thesis aimed to develop and validate custom tank with an open rectangular arena that allows free thermoregulation within a finely controlled temperature gradient lacking physical barriers. The tank maintained a stable uniform temperature differential of 6.4°C (18.5-24.9°C) for 48 hours.

Despite recent machine vision successes in applying conventional algorithmic and machine-learning approaches to the problem of tracking multiple targets in a visual scene, it remains a challenge to capture group behaviour from video while maintaining unique identities without physical markers. This thesis aimed to develop a markerless multitracking protocol which trains a convolutional neural network with the visual information of targets to create unique fingerprints that are matched to targets to correct tracking mistakes during complex social behaviour.

To demonstrate the application of this new approach, this thesis also presents two proof-of-concept trials examining how group size modulates the behaviour of goldfish (*Carrasius auratus*) shoals, and how febrile and afebrile fish mutually influence each other within a shoal. Larger shoals of goldfish preferred colder temperatures and swam slower than smaller shoals, while individual differences in group speed were more repeatable than differences in temperature preference. These preliminary findings argue that the methods developed herein are well-suited to provide insight into individual contributions to shoal movement and temperature selection. This work also represents a novel aquatic approach to study the contributions of fever to innate immunity and disease transmission.

Preface

This thesis is an original work by E. Proctor unless otherwise stated. This research project, of which this thesis is a part, received ethical approval through the University of Alberta Ethics Board. Animal care and use protocols for this project and thesis are under Protocol #706, “Comparative biology of fish phagocytic anti-microbial responses.”.

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List of Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
CFD	Computational Fluid Dynamics
CNN	Convolutional Neural Network
LMM	Linear mixed model
PPM	Parts per Million
PIT	Passive Integrated Transponder
QR	Quick-Response code
R	Behavioural Repeatability
RFID	Radio Frequency Identification
RTPT	Rectangular Temperature Preference Tank
SGD	Stochastic Gradient Descent
VIE	Visible Plant Elastomer

Chapter 1: Shoaling Behaviour & Thermoregulation in Fish

1.1 Abstract

Shoaling behaviour in fish is ubiquitous and confers survival advantages to the group yet remains poorly understood. Social groups are formed and dispersed via elective engagement in social behaviour driven by perceived benefits and risks by individuals. Shoaling behaviour is influenced by other factors such as behavioural thermoregulation that is critical to maintaining an optimal temperature range for physiological rates, hatching success, growth, and development of offspring. Investigating how fish groups collectively thermoregulate is essential to understand fish ecology and to assess disruptions to social behaviour caused by climate warming and anthropogenic stressors. Investigators studying collective thermoregulation have faced significant challenges that limit their ability to to reproduce natural thermoregulatory conditions and avoid behavioural biases within their experimental aquaria. This thesis research presents an experimental system which combines a novel experimental enclosure with an identity-based multitracking protocol designed to overcome methodological challenges. I used this system to conduct proof-of-concept trials examining group- and individual-level metrics during shoaling in a gregarious cyprinid.

In this chapter I briefly review the biology of shoaling and thermoregulation in ectotherms, both of these being extensive fields in their own right. I review the methodology previously used to study shoaling and temperature preference in fish. This chapter highlights methodological challenges I overcame and identifies gaps in the literature regarding how shoaling, individual personality, and temperature are likely to interact.

1.2 Fish ecology & behaviour

1.2.1 Introduction

Fish have adapted and thrived in nearly every aquatic environment on Earth. 35 600 species of extant fish have been identified, the true number likely closer to 40 000 (FishBase 2024). Fish habitats differ extraordinarily in their environmental conditions (temperature, salinity, pH, O_2, etc.) and spatial distribution (Bone & Moore 2008). For example, the desert pupfish (*Cyprinodon* spp.) are limited to solitary lakes and streams, while the bathypelagic stomiatoids (*Cyclothone* spp.) live worldwide from depths between 100m and 2000m and have a total population in the billions (Thompson & Kenchington 2017).

Space availability shapes predation pressure, a main driver behind behavioural adaptation (Ford & Swearer 2013; Cerini et. al 2023). Species living in dense and cluttered environments, such as reefs or kelp forests, will use their environment for camouflage and refuge (Ingrum et. al 2010). Species living in open water do not have the advantage of cover and are more vulnerable to large predators, thus engage in social vigilance (Kuruvilla et. al 2022). Dissecting the factors which underpin and modulate social behaviour is of keen interest in understanding the ecology of fish species. Social behaviour is complex and can rarely be resolved in the wild due to complexities within the natural environment and limitations in measurement techniques (Axelsen et. al 2001; Polyakov et. al 2022). These limitations have facilitated the use of specialized laboratory techniques to study social behaviour, with a trade-off that they typically fail to present realistic environmental choices to animals, nor do they resolve contributions from among-individual variation.

Given the ubiquity of social interactions across all domains of life, elucidating how rapid environmental change modulates social behaviour is critical to understanding the effects of anthropogenic environmental stressors on biodiversity (Fisher et. al 2021). Climate change is a current and prominent threat to global biodiversity and ecosystem health, with global temperatures predicted to rise 2-4 degrees by the end of the century (IPCC 2007). Agriculture, shipping, and urbanization introduce noise, pollution, eutrophication, and turbidity to aquatic environments, which are especially vulnerable as water stores much of the excess heat associated with climate change (Allibhai et. al 2023). Freshwater fish are physiologically optimized for thermo-stable environments, and thus are critically sensitive to temperature flux in their environment (Johansen & Jones 2011). Marine species may become displaced if significant thermal changes occur in their habitat, leading to shifts in geographical distribution of species (Perry et. al 2005). Thermal stress can limit the energy available for social behaviours or alleviate energetic constraints on costly social behaviours such as communication and aggression (Fisher et. al 2021). Studying the patterns and interactions that govern thermoregulation in social fish species may be critical to informing conservation strategies and tracking ecological shifts created by climate warming (Maldonado-Chaparro & Chaverri 2020).

1.2.2 Shoaling behaviour

Social behaviour is ubiquitous in fish (Keenleyside 1955) but the factors determining the degree of aggregation and group size in wild populations are difficult to discern (Polyakov et. al 2022). A shoal is defined as a social group of individuals that are co-located but may be engaging in different individual behaviours, such as foraging, vigilance, or communication (Pitcher 1986). Schooling is a form of shoaling in which the members are synchronized in their swimming speed and direction, while possibly engaged in cooperative behaviours (Magurran 1990). Elective membership in shoaling behaviour likely evolved as an evolutionarily stable strategy through the individual reassessment of benefits versus risks of remaining in the group. Benefits of shoaling and schooling include better vigilance and decision-making (Ward et. al 2011), more effective sampling of patchy foraging sites (Pitcher et. al 1982), defense from predators (Magurran 1990), hydrodynamic advantage (Li et. al 2020), and reduced stress (Nadler et. al 2016). Individual risks of shoaling include increased visibility to predators (Pitcher 1986), increased intraspecific competition for food (Polyakov et. al 2022), and increased transmission of parasites and disease (Walsman et. al 2022).

Accounting for the benefits and risks to an individual electing to participate in a shoal fails to explain group sizes seen in the wild, nor does it explain why many species school as juveniles but lose social tendencies into adulthood (Rieucau et. al 2014; Keenleyside 1955). Group structure in schooling also remains enigmatic; schools appear egalitarian as opposed to other vertebrate taxa where grouping tends towards a hierarchical structure (Pitcher 1986). Groups of fish make decisions faster and more accurately than solitary fish, though the mechanism is unclear (Ward et. al 2011). Dissemination of sensory information may enhance navigation and detection of threats rather than decisions made by a few key decision makers (Magurran &

Higham 1987). Developing methodological tools to resolve shoaling behaviour at the individual level will assist in closing knowledge gaps regarding collective behaviour.

1.2.3 Thermoregulation

Fish maximise their fitness via behavioural selection of temperatures optimal for growth and metabolism, a process termed thermal preference (Beitinger & Fitzpatrick 1979; Wallman & Bennett 2006). Ectothermic vertebrates do not expend metabolic energy to maintain a homeostatic internal temperature, instead their internal temperature and metabolic rate fluctuate with ambient temperature (Kuruvilla et. al 2022). Fishes living in thermally heterogeneous environments leverage thermal preference to behaviourally select positions in the water column within a thermal preferendum that optimizes growth (Krause et. al 1998), development, reproductive rate (Jonsson & Jonsson 2009), digestion (Elliot 1972), and immune function (Haddad et. al 2023). Thermal preference is dependent on rearing temperature and determines the geographical distribution of the species (Hoyland et. al 1979).

Quantifying thermal preference under dynamic temperature conditions will allow examination of physiological as well as behavioural responses to temperature. Temperature varies based on several factors depending the type of aquatic environment, including season, time of day, depth, changes in water flow, currents, and anthropogenic activity (Bartolini et. al 2015). Fish may swim faster in warmer water due to effects linked to muscle kinematics (James 2013). Indeed, activity seems to increase with temperature (Colchen et. al 2017), but its effect on swimming performance is species-specific. Tropical reef damselfishes (*Pomacentridae* spp.) have reduced swimming capacity in response to warmer water (Johansen & Jones 2011). The walleye pollock

(*Theragra chalcogramma*) decreases its routine swimming speed in response to increasing temperature while its maximum swim speed increases (Hurst 2007). This suggests behavioural rather than physiological regulation of swimming speed, but when and how fish choose to regulate their activity in response to temperature is still unclear.

Aside from thermal preference, temperature is well established as a behavioural cue in fish. For example, Atlantic salmon (*Salmo salar*) use a drop in water temperature as a cue to switch from diurnal to nocturnal foraging (Fraser et. al 1993). Behavioural responses to temperature may be dependent on individual personality (Rey et. al 2015), though little is known about the connection between personality traits and temperature (Biro & Stamps 2010). It is unclear whether temperature cues create switches in group dynamics, or whether individual responses to temperature are also true of groups.

1.2.4 Behavioural fever exemplifies the link between thermoregulation and fitness

Behavioural fever is an established example of the link between thermoregulation and fitness in fish (Boltaña et. al 2013; Rakus et. al 2017; Rey et. al 2017). During infection with a pathogen, fish can exhibit altered temperature preference which mediates certain innate immune pathways. Using an annular tank, Haddad et. al (2023) demonstrated that goldfish respond to *Aeromonas veronii* infection with a stereotypic behavioural response characterised by 2-4°C warmer temperature preference and pronounced lethargy. Compared to static temperature conditions, fish infected in a horizontal annular thermal gradient inclusive of the febrile temperature range had altered leukocyte inflammation, inflammation control, and tissue repair, which resulted in more effective healing and return to homeostasis (Soliman & Barreda 2022). Given that homologous hormonal and neuronal pathways regulate both endo- and ectothermic fever in vertebrates, behavioural fever represents an opportunity to further explore gaps in our knowledge of the innate effectors with which fever interacts.

Behavioural fever can be non-invasively measured by observing an organism's behaviour within an appropriate thermal apparatus, providing an opportunity to use recently developed machine vision techniques for automatically tracking behaviour in a group (Delcourt et. al 2009; Qian et. al 2016). In Chapter 3 the current study presents a method and proof of principle investigating disparate thermoregulatory programs in a group using behavioural fever in goldfish. Such investigation will enlighten gaps regarding how differing thermal strategies and individual variation contribute to group metrics during fish shoaling and schooling, or if behavioural modification influences disease transmission dynamics (Rey et. al 2015).

1.2.5 Temperature-dependent effects on shoaling

Like behavioural fever, thermal preference during shoaling and schooling remains understudied. Individual fish modify their behaviour to remain within their preferred thermal preference, but the link between individual variation and group thermoregulation is unclear. Social patterns in fish differ throughout the diurnal cycle and change seasonally in the wild, but quantifying the role of temperature in these patterns is challenging (Nakayama et. al 2016). Shoal cohesion is measured by averaging the pairwise nearest-neighbor distances between shoal mates to quantify how tightly packed individuals are within the shoal volume. Guppies (*Poecilia reticulata*) have been shown to shoal more cohesively at higher temperatures (Weetman 1998; Allibhai et. al 2022). Kuruvilla et. al (2022) found no effect of temperature on shoal cohesion during unperturbed swimming in golden shiners (*Notemigonus crysoleucas*). Juvenile walleye pollocks, brown trout (*Salmo frutta*) fry, and giant danios (*Devario aequipinnatus*), shoal less cohesively as temperature increases (Colchen et. al 2017; Bartolini et. al 2015; Hurst 2007). Some authors interpret decreased cohesion as evidence of social disruption caused by higher temperatures, while others estimate that increasing shoal volume represents an adaptive minimization of hypoxia while traversing warmer waters (Domenici et. al 2017). The relationship between temperature and shoal cohesion must be studied further to bring clarity to these findings.

To complicate matters further, temperature-dependent effects on shoal cohesion seem to be context-dependent. Presence of a predation threat influences the relationship between group metrics and temperature; despite there being no apparent influence of temperature on shoal cohesion during unperturbed swimming, shoal cohesion in golden shiners increased dramatically at higher temperatures in the presence of a loom stimulus (Kuruvilla et. al 2022). Zanghi et. al (2023) found similar results in guppies in the presence of their natural predator, the blue acara (*Andinoacara pulcher*), but did not measure shoal density during unperturbed swimming. The context under which group behaviour is occurring may have drastic effects on the optimal temperature for swimming or escape.

1.2.6 Effects of group size on shoaling

The degree of social interaction and elective group size may have dramatic effects on the flux of energy and materials through aquatic ecosystems (Gil & Hein 2017). Group size influences survival and foraging success in schooling species, despite group size being highly variable in the wild. For example, Polyakov et. al (2022) analyzed pacific salmon (*Oncorhynchus* spp.)

catch size as a proxy for group size and found that larger groups experienced lower predation risk. However, it depended on the species whether larger groups had increased foraging success, indicating that safety-competition trade-offs differ among species. Reef fish use information about density and activity of nearby conspecifics to decide when to feed or flee a food patch, and group size has a positive influence on the time individuals spend feeding (Gil & Hein 2017). The degree to which group size influences social thermoregulation, as well as the factors determining optimal group size in the wild, are still unclear.

1.2.7 Individual variation within social groups

Behaviours often show some degree of plasticity in conjunction with consistent individual differences, where the expression of behaviour depends on the environmental context (Bentzur et. al 2021). For example, guppies develop more stable and differentiated social connections under greater perceived predation risk (Allibhai et. al 2022). Thermal preference may be determined by personality traits; zebrafish (*Danio rerio*) exhibit higher activity at their individual preferred temperature, which may correlate with other behavioural traits (Rey et. al 2015). It remains poorly understood what factors drive group-specific behavioural patterns, and how individual differences contribute to group structure, decisions, and thermal preference.

1.3 Studying shoaling behaviour & thermoregulation

1.3.1 Studying shoaling behaviour

Investigators studying schooling or shoaling behaviours have utilized field studies, mathematical modelling, and laboratory approaches. Field studies are complicated and rarely permit an analysis of individual positions (Delcourt et. al 2006; Handegard et. al 2005). For example, fish schools can be tracked with sonar, but is limited to analysis of group-level effects (Axelsen et. al 2001). Filmed sequences in nature can rarely be analyzed automatically, and manual quantification of behaviour is labour intensive and prone to error (Biro et. al 2010; Weetman et. al 1998). Radio tracking, for example using passive integrated transponder (PIT)-tags, is limited to measurement frequencies between 1-10 minutes, and their detection by radio frequency identification (RFID) antennae is affected by cover, depth, and body posture (Bruyndoncx et. al 2002; Wallman & Bennett 2006). Visible implant elastomer (VIE) tags are widely used in biological research and are used to mark individuals in a fish school for tracking (Bruyndoncx et. al 2002). However, artificial tags are limited to a small number of individuals at a time and may influence fish behaviour, necessitating development of markerless tracking methods (see §1.3.4).

Laboratory models have delivered the most consistent and replicable results for analyzing social behaviour, based on the ability to control and manipulate environmental variables, isolate specific behavioural syndromes, and produce replicable results across trials (Christensen et. al 2021; Egan et. al 2009; Bierbach et. al 2017). Mathematical approaches also hold promise in modelling schooling behaviour and responses to shifting thermal environments (Viscido et. al 2004; Koenigstein et. al 2016; Walsman et. al 2022) but require a comparison with empirical results to determine the degree of validity for each model. Social metrics are sensitive to methodological differences; how observations are made should be always expected to play a role in shaping social behaviour (Ogino et. al 2023). Observations may be biased towards more easily identified individuals. For example, in the current study, darkly coloured goldfish are much harder to distinguish from the background and are thus poorly tracked. A drawback of laboratory studies is that the interactive effects of multiple biotic and abiotic factors becomes difficult (Christensen et. al 2021).

1.3.2 Tank systems

Various experimental designs have been developed to study temperature preference in fish, which can be grouped into vertical gradient, horizontal gradient, and shuttlebox systems (Christensen et. al 2021). Vertical gradients take advantage of the stratification of temperature in a deep thermal column but are confounded by differentials in light and pressure (Garside & Tait 1958; Kwain & McCauley 1978; Lafrance et. al 2005). Horizontal gradients use a single chamber or series of connected chambers with water flow directed away from a single heat source (Rey et. al 2015; Lafrance et. al 2005; Mathur et. al 1982; Wallman & Bennett 2006). These systems often present a series of discreet temperature choices to the animal rather than a continuous thermal gradient; selection with these conditions may require alternative decision processes than navigating a thermal field by kinesis (Lagerspetz & Vanio 2006). Linear flow in the same direction as the gradient may also create selection asymmetry depending on the direction of movement due to flow taxis. Shuttleboxes contain two chambers that the organism shuttles between to maintain their preferred temperature (Neill et. al 1972; Boltana et. al 2013; Macnaughton et. al 2018). Determining thermal preference using a shuttlebox requires conditioning of the organism to switch chambers when the temperature becomes unpreferrable. This methodology may be limited in broad applicability due to differences in learning capacity between taxa, raising the question whether natural thermoregulation can be represented by a highly controlled, conditioned response (Lagerspetz & Vainio 2006).

One possible source of error in horizontal gradient systems is the use of a box- or tube-shaped apparatus, in which individuals of some species (or some individuals within a group) may aggregate in the corners of the box or ends of the tube (positive thigmotaxis), while other species may tend to avoid these positions (negative thigmotaxis) (Lagerspetz & Vainio 2006). Positive and negative thigmotaxis may create behavioural responses driven by preference or avoidance of corners and edges rather than the environmental gradient being tested. This bias may be negligible in simple aquaria but becomes amplified in a system in which multiple tanks are joined together using tunnels, which introduces tight spaces, edges, and corners.

Shuttleboxes and horizontal gradients are still the prevalent method of examining thermal preference in fish today, but these systems are confounded by additional variables. These systems suffer from offering only a limited number of discreet choices of temperature that are separated by barriers. Thermal choices can be affected by thigmotactic orientation due to the uneven distances to corners and walls (Lagerspetz & Vainio 2006). Flat edges and physical barriers present in these types of aquaria can be perceived as cover and prevent active searching for alternative temperatures. They may allow a range of water temperatures to be established, but they contain variable depth and currents that can play a role in animal positioning within the apparatus.

Annular chambers have the advantage of presenting the same range of temperatures to all experimental animals. Site-specific bias created by positive or negative thigmotaxis, uneven light conditions, and pressure can be avoided. It is possible to generate predictable currents and mixing of water, as well as providing a sufficiently deep water column while preventing thermal stratification. Improving upon earlier annular designs, Myrick, Folger & Cech Jr (2004) developed a system with constant flow rates and temperature, allowing a wide yet fine scaled gradient suitable for eurythermal species. The swim chamber is a taurus consisting of eight continuous segments which are fed by an outer inflow ring. Water flows unidirectionally from the outermost ring to the swimming chamber and lastly to the drain through pores, generating eight temperature zones sustained by fluid dynamics, providing an environment for fish to deploy behavioural thermoregulation by freely translocating to any of the overlapping temperature zones. This allows continuous resolution of behavioural thermoregulation beyond just a distinct choice of cells by taking advantage of the barrierless thermal zones and continuous behavioural tracking throughout the experiment. Day and night are both captured with equal contrast by recording behaviour using an infrared camera.

Annular aquaria provide exceptional behavioural resolution and more realistic choice to the animals but are not ideal for capturing social behaviour (Haddad et. al 2023). Individuals exhibiting distinct thermoregulatory programs cannot communicate or visually identify each other. Additionally, the inner surface of the swim chamber occludes individuals from view of the camera if they are up against it. This causes problems with group tracking (see §1.3.4). Andreassen (2019) developed a version of the annular tank that prevents this by setting the inner wall of the swim chamber at an angle but the chamber is space restrictive and lacks the range of thermal choice required for eurythermic fish.

The current study proposes a novel horizontal tank design which makes improvements upon the existing models mentioned above. It features a horizontal gradient that is formed transversely to the input flow to reduce bias with respect to temperature selection and movement direction (see §2.3). The swimming arena is an open rectangle which minimizes environmental occlusions, reduces behavioural bias from positive and negative thigmotaxis, and allows a suitable arena for social interaction while decreasing the number of frequent occlusions. The gradient is created with five overlapping thermal zones that provide a continuous temperature gradient free of barriers. The design has several advantages compared to previous works regarding the characterization of the impacts of temperature upon shoaling behaviours.

1.4 Studying shoaling behaviour using video tracking

Video tracking is a recently adopted and powerful method of extracting behavioural information from visual data (Mathur et. al 2011, Egan et. al 2009). Visually tracking each fish in a large school is an effective way to quantitatively investigate behaviour patterns and underlying rules governing group structure (Wang et. al 2017). Horizontal experimental aquaria that are relatively shallow are perfect for top-view video and Two-Dimensional (2D) tracking. However, it is challenging to reliably retain individual identities while observing a group of tracked objects (targets) that are non-rigid and move unpredictably (Butail & Paley 2010). Multitracking software holds promise to address this challenge.

Multitracking software use statistical algorithms to identify and predict the movement of multiple targets. Behavioural tracking algorithms capture the information in a scene by making measurements (detection) and assigning the correct measurements to each target using a model (assignment). In a complicated scene, there are usually many measurements made but only a few targets. The measurements are filtered and assigned to targets using a rule such as nearest neighbor (Bar-Shalom et. al 1990), probabilistic data association (Bar-Shalom & Tse 1975), or multiple hypothesis tracking (Blackman 2004). The measurement-target matches are integrated into a dynamic state model which is then used to estimate the likely positions of targets when the scene is iterated through time (Arulampalam et. al 2002).

1.4.1 Occlusions limit tracking fidelity

Researchers determining animal trajectories from video recordings face the problem of maintaining correct animal identifications after individuals touch, cross, or are obscured from vision by the environment (Dolado et. al 2015). Mutual occlusions arises when the trajectories of two targets (eg. fish) cross, or the targets overlap for several time units in succession, which causes the targets to merge for the duration of the occlusion (Delcourt et. al 2013). Due to the merging of the targets, it is difficult to reassign correct identities after the occlusion. As a result, these occlusion events cause assignment errors in which the individuals are misidentified or lost altogether. As environmental complexity and target density increases, the number of occlusions increases exponentially, and thus the difficulty in resolving assignment errors. In an automated tracking system, these errors will accumulate over time without manual corrections, and cause the identities to be fully shuffled over a sufficiently long period of time. If the goal is a rigorous analysis of the individual behaviours then perfectly maintaining identities is a requirement. This limits the duration of analysis that can provide reliable results.

One method for avoiding such errors and allowing for long-term tracking of uniquely identified targets in complex visual environments is to use markers that can be uniquely identified by computer vision systems (Crall et. al 2015). These could be a unique colour marker in the form of a coloured bead (Delcourt et. al 2013), a barcode or Quick-Response (QR) code (Crall et. al 2015), or a visible implant elastomer (VIE) tag (Bruyndoncx et. al 2002). However, these tags, particularly those that require an invasive insertion into the skin, may cause inflammation and alter the natural behaviour of animals (Switzer & Combes 2016). Moreover, bioethical considerations favour a less invasive approach. Therefore, there is incentive to develop markerless tracking systems that are still able to uniquely identify individuals. Finally, it is important to consider that these methods do not assist in detection during occlusion events where the tag is obscured.

1.4.2 Appearance-based tracking

These difficulties have motivated investigators studying social behaviour to apply image recognition techniques based on handcrafted or learned imaged features to identify each individual in a fish group during tracking. Pérez-Escudero et. al (2014) introduced a method in which a ‘fingerprint’ is generated for each individual by sampling a set of reference images from the video and aggregating those with the highest probability of similarity. The reference images are transformed into 2D maps based on pair-wise distances between points and differences in colour intensity. 2D fingerprints are compared against frames before and after mutual occlusions during a separate analysis pass to correct assignment errors. This method, and similar approaches to track unmarked individuals (Dolado et. al 2015; Rasch et. al 2016; Rodriguez et. al 2017; Wang et. al 2017) have been successful in tracking groups of 2-20 individuals over short durations. By extracting and learning the features of the tracked targets and then identifying each detected target in each frame, tracking by recognition strategy enables a tracking system to preserve the individuals' identities even after trajectory interruption due to occlusion. However, as the complexity of the environment increases, available space decreases, or the number of individuals increases, the number of occlusions increases exponentially, and thus the difficulty in resolving assignment errors. The methods above also use significant processing power, creating limitations in measurement frequency (xyTracker, 40Hz; ToxTrac, 25Hz), number of individuals tracked simultaneously, and total analysis time.

1.4.3 Tracking enhancement using neural networks

Machine learning is critical to many automated detection and recognition tasks in our technological society (LeCun et. al 2015). Neural networks can perform recognition tasks that are extremely sensitive to minute details but insensitive to variation in irrelevant features. This is done by a process called representational learning, in which raw data is automatically processed and classified into increasingly abstract and complex representations required for recognition. A convolutional neural network (CNN) is a type of representational network with a specialized structure that takes advantage of natural patterns in many types of data to enhance feature detection. Convolutional networks are particularly useful in learning tasks which require a large, heterogeneous training databases and require recognition of local features, motifs, and groups of symbols. Thus, CNNs have been widely used in recognition tasks such as speech recognition, handwriting recognition, and face recognition (Cadieu et. al 2014).

Recent developments in machine learning have enhanced machine-vision techniques in the field of animal tracking. For example, Romero-Ferrero et. al (2019) introduced idtracker.ai, an iteration upon idTracker (Pérez-Escudero et. al 2014) which implements a learning procedure using two CNNs that automatically detect the presence of occlusions and identify each individual between occlusion events. idtracker.ai shows vast improvement in speed and identification accuracy over idTracker and allows tracking and automatic identification of up to 100 individuals. The current study takes advantage of TRex (Walter & Couzin 2021), an open-source identification-based multitracking software that follows Romero-Ferrero et. al (2019). Similar to idtracker.ai, TRex can automatically learn the identity of up to 100 individuals by training an artificial neural network to visually differentiate between individuals. TRex outperforms idtracker.ai in accuracy and computation speed at small group sizes, and achieves a similar high level of accuracy with reduced computing requirements at large group sizes (Walter & Couzin 2021).

1.4.4 Supervised learning

Training neural networks requires a sufficient amount of high quality and varied training data as well as an efficient training method that minimizes manual engineering of feature detection (LeCun et. al 2015). Stochastic gradient descent (SGD) is a procedure commonly used among deep-learning practitioners that takes advantage of the stochastic nature of small sets of training examples. The input-output function of a neural network is determined by millions of internal adjustable parameters called ‘weights’. During SGD, the machine is shown many small sets of labelled training examples for which we know the desired output, and a set of weights is found that minimizes the error between the real output and the desired output. After each step of training, a gradient vector is computed which indicates the change in weights which will lead to the ‘steepest descent’, or the quickest path, to a local minimum in the average error. This procedure quickly finds a good set of weights compared to more elaborate optimization techniques (LeCun et. al 2015).

1.5 Summary

Fish taxa exhibit astounding variation in the prevalence and ecological function of their social behaviours. Social behaviour in fish is governed by a trade-off between risks and benefits of elective membership. Individuals may sacrifice their individual foraging efficiency to take advantage of the safety of the group, or risk venturing on their own to prioritize thermal preference or reproduction. Group size in the wild is determined by a variety of factors including season, perceived risk of predation, intraspecific competition, and energetic availability for individualized behaviours such as courting, communication, and reproduction. Social groups persist despite the coexistence of distinct coexisting evolutionarily stable behavioural strategies.

Temperature is the primary abiotic factor modulating the energetics of aquatic ecosystems and affects individual behaviour as well as predator-prey relationships. The influence of temperature on shoaling behaviour in fish is still poorly understood due to experimental limitations. Studies on thermal preference require specialized aquaria that have previously been limited in resolution of environmental and behavioural variables. Increasing temperature has been found to increase shoal cohesion in guppies but had the opposite effect in brown trout fry and giant danios. In the context of a predator threat, increasing temperature had an enhanced effect on increasing shoal cohesion in guppies as well as golden shiners. In golden shiners, increasing group size dampened the effect of temperature on group metrics. Overall the literature has highlighted species- and context-specific effects of temperature on shoaling behaviour with varying levels of confidence and resolution.

Furthermore, studies that consider among-individual differences in shoaling behaviour have remained challenging, but artificial intelligence options are enabling new approaches that require refinement. This will allow various gaps in understanding to be filled, including the focus here on how water temperature and shoaling behaviours interact.

Goldfish are eurythermic cyprinids that have demonstrated practical use as a behavioural and immunological model but have not been studied in the context of shoaling behaviour. The goal of this thesis research is to characterize the effect of temperature on shoaling behaviour in goldfish using a novel experimental system which allows free thermoregulation and high-fidelity behavioural resolution.

Chapter 2: Materials & Methods Development

2.1 Abstract

Social behaviour in fish is of great interest to ecologists and climate scientists, particularly how shoaling is disrupted or modulated by anthropogenic environmental stressors. Current laboratory methods to study shoaling behaviour in fish are specialized, such that producing replicable results between studies and eliminating behavioural biases is challenging. Aquaria designed to measure temperature preferences in fish fail to provide realistic environmental gradients to animals, restrict social behaviour through visual occlusion and tight spaces, and ignore systematic biases in an enclosed environment created by thigmotaxis and rheotaxis. This chapter outlines a behavioural analysis protocol including a novel experimental aquarium designed to provide continuous temperature choice while eliminating systematic behavioural biases. Next, it provides a behaviour tracking method using deep learning to enhance markerless visual identification of individuals. The chapter concludes with an evaluation of the current system and proposes improvements and future developments in studying social thermoregulation.

2.2 Introduction

Motile organisms actively choose environments that are physiologically favourable and avoid those that are averse (Wallman & Bennett 2006). Selection of environments within a preferred range can be viewed as behavioural manifestations of an animal's physiological response to their environment. Recently, preferred environmental ranges by animals have also been incorporated into modelling of species distribution and responses to environmental change (Pont et. al 2015).

Environmental preference and avoidance can be studied in laboratory settings with the basic idea of presenting an animal with an environmental gradient and assessing its choice. In controlled laboratory experiments, environmental preference and avoidance levels can be determined while excluding potential confounding factors, though studying interactive effects of multiple biotic and abiotic factors becomes difficult (Zanghi et. al 2023).

A common experimental setup for thermal preference involves multiple chambers heated to different temperatures that the animal must shuttle between to exert thermal preference (Lafrance et. al 2005; Rey et. al 2015; Macnaughton et. al 2013). These systems present a discreet number of choices to the animal rather than a continuous thermal gradient in which they would normally be able to express thermal kinesis and behavioural selection. Having multiple chambers connected with portals introduces tight spaces and flat surfaces that introduce thigmotactic bias and induce cover-seeking behaviour that confounds the actual selection in the environmental gradient (Lagerspetz & Vainio 2006). Fish locomoting between multiple chambers are difficult to keep under continuous observation, which is critical when capturing the behaviour of multiple individuals. Many thermal preference systems are designed for a single focal individual and do not facilitate collective movement of groups through tight spaces, nor do they allow appropriate visual propagation of signals that are key to certain social behaviours (Ward et. al 2011).

Annular arenas have achieved success in ameliorating issues in behavioural analysis created by horizontal gradient and shuttlebox systems (Myrick et. al 2004, Haddad et. al 2023). The annular arena shape limits behavioural edge-effects from corners and allows fine temperature control over each section of the ring, producing a stable and wide-range gradient. However, the shape is space limiting, restricting the size of the fish that can comfortably move within it, preventing fish exhibiting distinct thermoregulatory programmes from seeing each other, and completely or partially occluding individuals from overhead observation. These limitations make the annular arena unsuitable for studying shoaling behaviour.

The current study introduces a tank, described in the following section, which is capable of producing a linear thermal gradient within an open rectangular arena, ameliorating issues with space, occlusion, and visual fields.

2.3 Animals

Common goldfish (*Carrasius auratus auratus*) at approximately 10-15cm in length, mix sex, were purchased from Mt. Parnell Fisheries (Mercersburg, PA, USA) and imported to Canada via Aquatic Imports (Calgary, Canada). Fish were held at the Aquatic Facility of the Department of Biological Sciences in 16°C ,opaque, continuous flow-through aquaria with a simulated natural photoperiod of 12 hours light alternating with 12 hours darkness. Fish were continually monitored for signs of disease and were fed once daily with floating pellets containing crude protein, fat, fibers, vitamins, and minerals (0060832; Mazuri Exotic Animal Nutrition, St. Louis, MO, USA). Water quality parameters were sustained at 5.5-6.5 PPM of dissolved oxygen and pH of 7.2-8.0. Before experimental handling, animals were randomly netted and anesthetized in a 50mg/L tricaine methanesulfonate solution (02168510; Syndel, WA). Fish were acclimated for at least 14 days preceding their use in experiments.

2.3.1 Ethics statement

All animals used in these experiments were maintained according to the guidelines of the Canadian Council on Animal Care (ACUC-Biosciences Protocol 706). The University of Alberta Animal Care and Use Committee approved all the protocols and specific training to perform the experiments. Fish were euthanized via cervical dislocation after anesthetization using tricaine methane sulphonate (TMS; 02168510; Syndel, WA, USA) and approved procedures. Procedures were designed and carried out to minimize animal stress and pain.

2.3.2 Self-resolving model of zymosan-induced acute peritonitis

Goldfish were challenged *in vivo* via intraperitoneal zymosan injection to examine behavioural segregation of febrile and afebrile individuals in a group. Zyomsan is a non-infectious, non-toxic pyrogen made up of β-glucans and other protein-carbohydrate complexes isolated from baker's yeast (*Saccharomyces cerevisiae*) (Di Carlo & Fiore 1958). Zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich, Z4250-250MG) was resuspended in phosphate-buffered saline and diluted to create a 25mg/mL working stock. Fish were anesthetized using a tricaine methane sulphonate solution (02168610; Syndel, WA, USA), and 2.5mg of zymosan was injected within a 100μL volume through the soft tissue under the left pectoral fin using a 25-gauge needle. This concentration was previously determined to promote a self-resolving acute inflammatory process where initial induction and subsequent resolution phases could be examined (Haddad et. al 2023). The febrile window lasted from 2-22 hours post injection, during which the fish preferred hotter temperatures (1-2°C above control) and exhibited a marked lethargy response in which their overall speed and transitions between thermal zones was decreased.

2.3.3 Examining cross-modulation of behaviour in mixed afebrile & febrile groups

To examine the effect of behavioural fever (see §3.4) on the behaviour of heterogeneous groups of fish containing both febrile and afebrile cohorts, I tracked six groups of 20 goldfish that contained different proportions of afebrile to febrile fish (afebrile:febrile; 20:0, 16:4, 12:8, 8:12, 4:16, 0:20). I injected fish in the febrile treatment with 25mg zymosan as described above. I did not inject fish in the afebrile treatment, as it was previously determined that uninjected goldfish behave identically to those injected with a control buffer. The fish were marked based on their treatment with a coloured elastic ring inserted in front of the tail fin. The groups were acclimatized to a linear 18.5-24.9°C thermal gradient within the Rectangular Thermal Preference Tank Version 2 (RTPT-2; see §2.4) for at least 1hr before behavioural observation. I recorded 2hr videos of the groups using an infrared camera (VICTOREM 262G41; NIR-Enhanced Monochrome Lens, IO Industries Inc., London, Ontario) and tracked the videos with TRex (v1.1.9) using the protocol described in §2.5 (uniqueness =0.67±0.1; see §2.6.1). I found the mean temperature preference (°C) and speed (cm/s) of each cohort over the 2h period by sampling the median temperature and speed per minute for each individual.

2.3.4 Examining the effect of group size on temperature preference and speed

I examined the effect of group size on the group-level metrics of temperature preference and speed using the RTPT-2 and the tracking protocol in TRex described in §2.5 (see Fig.2.6a for uniqueness data). I acclimatized groups of 4, 8, 12, 16, and 20 untreated goldfish in an 18.5-24.9°C linear thermal gradient within the RTPT-2 for at least 1hr before recording their unperturbed swimming for 30 minutes with an infrared camera (VICTOREM 262G41; NIR-Enhanced Monochrome Lens, IO Industries Inc., London, Ontario).

2.3.5 Statistical analysis

I analyzed temperature preference and speed data from the experiment above as response variables in separate linear mixed models (LMM) using the lme4 package v.1.1-35.1 (Bates et. al 2014) in R Studio (v.2023.12.1+402). I used median temperature per minute and median speed per minute in the models, as those data were well distributed and demonstrated meaningful changes between repeated observations. I partitioned the total behavioural variance into variance dependent on FishID (among-individual variance) and the remainder (residual variance). I found the behavioural repeatability using the ratio of among-individual variance to the total variance (Bell et. al 2009). For each group size, I constructed an LMM of the form X~1+(1|FishID), where X is the response variable and FishID is the grouping factor (Bates et. al 2014). This model estimates a random intercept for each level of the grouping factor. I generated 95% confidence intervals by simulating the model using Markov chain Monte Carlo estimation.

I created a global LMM encompassing all group sizes of the form X~Group Size+(1|GroupID), where X is the dependent variable, GroupID is the grouping factor, and Group Size is a fixed factor. GroupID is a composite variable that uniquely identifies each fish and its group. I partitioned the total behavioural variation into its components and calculated repeatability as above.

2.4 Rectangular Thermal Preference Tank (RTPT)

The RTPT is a specialized behavioural preference enclosure with the ability to precisely control the flow and temperature of each of the distinct temperature inputs. The input manifold follows the design of the annular tanks designed by Myrick et. al (2004) and Haddad et. al (2023), but here I favoured an open rectangular arena as opposed to an annular design. The arena is fed by ten paired inflow channels (Fig. 1b). I set the paired inflows to five distinct temperatures, creating overlapping thermal zones maintained by flow transverse to the gradient direction.

The tank measures 74cm by 134cm and is 28cm deep. The entire chamber is mounted on a black acrylic base and the walls are made of clear acrylic that is 1.5cm thick. The interior arena measures 60cm by 120cm. Inflow segments border the long edges of the arena and drainage segments are attached to each end (Fig. 2.1a). The drainage segments are 20cm wide and are each divided into three channels with their own drain. Each drain can be fitted with a PVC pipe of variable length to control the drainage height, which allows variation of the water depth within the inner arena. Water drains from the arena through pores communicating with the drainage channels at a height of 2cm from the tank base (Fig 2.1a). The water depth within the tank was maintained at 18cm during all the trials conducted.

The two inflow segments are each divided into 5 channels, each with 5 equidistant pores placed 18cm from the bottom of the tank (Fig. 2.1a). Hydrostatic pressure forces water through the pores into the inner arena as the channels are filled.

2.4.1 Design & development

The RTPT underwent multiple stages of development. Version 1 (RTPT-1) was constructed based on a design created by an undergraduate mechanical engineering team (Deep Blue Consulting, University of Alberta. See appendix). The tank was built to specification in the Biology Fabrication Shop.

The thermal gradient had been estimated using finite point analysis and computational fluid dynamics (CFD) analysis but required functional testing and adjustment to achieve a successful gradient. I established a water distribution system to feed the inflow channels using valves and flexible PVC tubing. This system connects the tank to the water input manifold and allows independent control of flow into each of the 10 inflow channels, ensuring the thermal zones are balanced between the two sides (Fig. 2.1c).

The RTPT-1 development goal was to establish a gradient with at least 8°C temperature difference between the warmest and coldest zone. A stable gradient was achieved by adjusting the input pressure, water height and zone temperatures. Electronic thermometers (12-Bit Temperature Smart Sensor, Onset Computer Corporation) and a mobile thermal camera (FLIR ONE Gen 3, Teledyne FLIR LLC) were used to monitor the gradient and capture thermal images and videos (Fig. 2.2). RTPT-1 successfully maintained a temperature differential of 5°C between the two extremes, but the interior temperature zones were poorly formed due to circular flow in the central region caused by flow along the tank edges and early diffusion of input flow (Fig. 2.2b). I also observed that heat was being transferred through the walls between the inflow channels, causing dilution of the input temperatures and limiting the overall temperature differential.

These issues prompted development of a second version (RTPT-2) in which the tank was rebuilt with modifications. Changes were identified from the results of long-form stability tests, thermal images, and CFD analysis by undergraduate colleague Massiel Copara Chino (Fig. 2.3). Key modifications included thermally isolating the inflow channels by thickening the acrylic borders, tightening the distribution of inflow pores to better direct input flow, and adjusting the walls to ensure equal sizes of inflow channels. We implemented vertical baffles to the sections bordering the inflow channels in order to dampen hydrodynamic forces and slosh-motion amplitudes (Ibrahim 2005). CFD analysis estimated improved distinction between the thermal zones using baffles even just 1cm in length (Fig 2.3b). During testing, a baffle length of 5cm demonstrated the greatest improvement to the gradient and disrupted circular flow in the interior region arena (Fig 2.3a). In the final RTPT-2 design, I chose baffles that were 5cm long and 1cm thick. The baffles were constructed from clear acrylic and attached bilaterally at the borders of each inflow channel (Fig 2.1c).

I oversaw the modification and design of RTPT-2, which was assembled in the Biology Fabrication Shop. The RTPT-1 water distribution system lacked stability and adequate spacing of valves, which allowed bubbles to form in the flexible tubing, greatly disrupting flow and gradient stability. I designed a mount for the tubing and valves that allowed convenient flow adjustments and prevented formation of bubbles. The mount was built in the Dept. of Biolgoical Sciences

Fabrication Shop and assembled with RTPT-2 in its final location in a biological containment room within the aquatics facility.

RTPT-2 maintained a differential of 6.4°C (24.9°C at the hottest, 18.5°C at the coldest) between the two extremes of the gradient and remained stable over a 48h period (Fig. 2.4b). I validated the stability of the gradient using two 12-bit electronic thermocouples situated in the center of each zone, offset by 6cm from the tank edge (Fig. 2.4a). I recorded temperature information using the HOBOware U30 data-logger (Onset Computer Corporation, Bourne, MA). To visualize the thermal surface, I assembled electronic thermocouples in a line and recorded the temperature in equidistant locations along the edge of the tank for 30 minutes, then shifted to capture the entire surface.

2.5 Multitracking with TRex

Having established a suitable environment for our thermoregulatory study, I sought to develop methods that would allow tracking of individual fish during the complex situation of shoaling. Maintaining individual identities while multitracking a fish shoal is paramount to the analysis of individual variation and classifying distinct behavioural programs within a group (Ogino et. al 2023). Recent developments in machine vision have made it possible to track multiple deformable targets that move unpredictably (Dolado et. al 2015; Rasch et. al 2016; Wang et. al 2017). Tracking fish shoals is a complex problem due to their tendency to mutually occlude each other from visual observation (see §1.4.1); this presents additional difficulties when examining social behaviour in which the individuals are in close proximity (Delcourt et. al 2013; Handegard et. al 2005). Mutual occlusions create assignment errors which, if propagated through time, end up fully shuffling the identities of the tracked targets (Rodriguez et. al 2017).

I established a protocol to capture and analyze behaviour in a goldfish shoal at the level of individual animals using the open-source 2D multitracking software TRex (Walter & Couzin 2021). TRex resolves assignment errors using a method introduced by idTracker (Pérez-Escudero et. al 2014; Romero-Ferrero et. al 2019); the unique 'fingerprint' of each individual is generated by training a CNN to recognize their visual appearance. These fingerprints are then compared against frames before and after mutual occlusions during a separate analysis pass to correct assignment errors.

The following sections briefly outline the multitracking protocol in TRex. I developed this protocol in multiple stages using a variety of testing conditions. The final result was achieved by incorporating feedback from discussions with the software developer and post-processing in Python.

2.5.1 Development

In the first development stage, I tracked groups of up to 8 untreated goldfish in an annular tank (Haddad et. al 2023). These preliminary trials had poor individual resolution due to occlusion by the circular walls of the tank which prevented accurate identity assignment. I henceforth carried out trials in the incomplete RTPT-1, which still lacked temperature regulation and flow-through capacity. I used 4 non-living robotic fish (ZURU Toys Ltd., Shenzhen, China) that automatically start swimming when introduced to water and locomote in wide circular paths using a small motor and battery. The robotic fish are 7cm long and weigh 50g. I chose to use robotic fish because they are convenient tracking targets; they swim in slow, predictable paths, have distinct patterning and colouring, and are naturally buoyant. I captured 30 minute videos of the robotic fish swimming in the RTPT-1, and TRex was able to track the 4 targets simultaneously (uniqueness >0.9, see §2.6.1). However, the duration of behavioural analysis of the robotic fish was limited to <30 minutes due to their proclivity to get stuck against tank edges and corners.

In the next stage I tracked live groups of goldfish in the RTPT-1. I established an 80L recirculating fish tank in the lab space which was filled with dechlorinated aquarium water and housed 60 goldfish while supervised during the day. I sampled groups of up to 20 fish from the recirculating fish tank to track for short durations (<1 hour) in the RTPT-1. The fish swam unperturbed in the RTPT-1 filled with 16°C aquarium water. The arena was aerated with bubble columns. These trials provided valuable data with which to refine the tracking protocol using live targets. Live goldfish were more challenging targets to track than the robotic fish, being larger and more deformable, as well as having the ability to change depth and swim underneath each other.

I finalized and validated the protocol in the RTPT-2 which I installed in a biocontainment room in the aquatics facility. I tracked groups up to 20 goldfish in the flow-through thermal gradient. With the refined protocol, TRex tracked small groups successfully, but I observed a drop-off in tracking quality as group size increased (see §2.6.1).

2.5.2 Segmentation

Segmentation is the first step in the tracking protocol once the video is captured. The video is segmented into background and foreground objects, the latter being the objects to be tracked, termed ‘blobs’. The TRex software package contains the task-specific tool TGrabs, which is primarily designed to perform segmentation and achieve real-time (online) tracking but lacks the graphical user interface and visual data tools that TRex provides. The fast online tracking algorithm uses information about the kinematics of each fish to maintain their identities, which is useful in making assessments about group properties (group centroid, alignment of individuals, density, etc.). When making conclusions about individuals instead, maintaining identities perfectly throughout the video is a critical requirement.

In this stage, I used TGrabs to perform segmentation, converting the video to a non-proprietary video format (.pv). I chose the background-subtraction method of segmentation, which separated the lighter blobs from the dark background using a specified threshold and constrained blob size parameters. I used TGrabs to crop out unnecessary parts of the recorded frame that would otherwise interfere with analysis. During segmentation the software is very sensitive to reflections or water disruptions that may be interpreted as blobs, so I spent time optimizing the lighting and exposure to achieve a quality conversion. I lit the arena with six infrared lamps (ICAMI IR Illuminator, Shenzhen Cany Electronics Technology Co., Ltd.; 48 LED IR Illuminator, Pomya Manufacturing Co., Ltd.) which I combined with a non-reflective black cardboard light shield to achieve maximum uniformity and consistency.

2.5.3 Tracking

Once I preprocessed the video with TGrabs, I adjusted tracking parameters using the visual tools in TRex to fine-tune the kinematic tracking of the individuals. TRex uses the same kinematic tracking procedure as in TGrabs. The algorithm generates trajectories based on a fixed number of individuals reported during the segmentation stage. Each trajectory is assigned to blobs using a probabilistic state-estimation model similar to a Kalman filter (Walter & Couzin 2021; Arulampalam et. al 2002). At this stage, the trajectories are divided at frames where individuals touch or occlude each other, as there may be a high degree of assignment error. I used the visual tool *track_ignore* to exclude problematic areas of the arena that contained reflections or disruptions, preventing them from being classified as blobs.

2.5.4 Visual identification

To correct assignment errors without the need for visually marking the individuals, TRex uses a CNN which automatically trains itself with segments of the video to be tracked. This machine-learning approach, termed visual identification, can learn the identities of up to 100 unmarked individuals based on visual appearance (Walter & Couzin 2021). Visual identification is the most processing-heavy (and therefore time-consuming) step, in which all previously collected tracking-related data is utilized to train the network to detect possible assignment mistakes. In addition, appearance data is collected from continuous segments of the video where all the individuals are visible and separated from each other.

The visual identification process is automatic, but I spent much of my time optimizing the previous steps to ensure that quality trajectories were produced before training could begin. To ensure the individuals were recorded with enough resolution and clarity irrespective of their position in the RTPT-2, I used a 2000x2000 pixel resolution camera with an infrared lens (VICTOREM 262G41; NIR-Enhanced Monochrome Lens, IO Industries Inc., London, Ontario) and a high-throughput data transfer module (DVR Express Core 2, IO Industries Inc., London, Ontario) which allowed capture 24h of 30Hz video (~14TB) in one recording session before being exported using high write-speed RAID hard-disk coupling and 1080p high-quality Mpeg compression which reduced file sizes with minimal artefacting.

2.5.5 Post-processing

After visual identification was completed, I exported the position and speed of each individual, per frame, in a numpy data-container format (.npz). The raw output from TRex contained missing data points and artifacts which I corrected with a post-processing protocol in Python (v3.11.6; Sypder v5.4.5), found in its entirety on GitHub at the link in found in Appendix 2.1.

The post-processing protocol aims to reduce discontinuity in the path of each fish while accounting for losses in detection and misassignment of blobs (Fig. 2.5). I developed a function which eliminates erroneous segments of the path while preserving true data points and allowing the path to be discontinuous during lapses in detection. Tracking artifacts tended to arise during situations where individuals touched or occluded each other, in which case the targets merged into a single blob. Identity assignment is limited to a single target per blob; after a certain number of frames following merging (determined by *track_max_reassign_time*) the tracker attempts to reassign one or both individuals to other blobs within the arena. If there are any erroneous blobs detected in the arena at that moment, such as shadows, reflections, or disruptions in surface of the water, one or both individuals may be misassigned until the targets separate.

These events appear in the path as instantaneous jumps to a distant point for a few frames, usually on the edge of the arena, before returning to the true path. The function reconstructs the path with only valid segments, which are defined as those that contain a certain threshold number of data points (l) that are within a tiny distance r of each other, where r represents the maximum distance a fish can travel between frames.

Using Python, I loaded the x coordinate (x), y coordinate (y), and speed (v) columns as lists from the .npz file exported by TRex. I treated each triple pair (x,y,v) as a single data point along the fish's path. I deleted all data points containing an .inf value, which indicates missing data. I then applied the pathFilter3 function, which created continuous segments by iterating through the path, concatenating those segments that were equal to or longer than the threshold length (l).

pathFilter3 first creates a new segment at index i (the first segment begins at index 0). The segment is initialized with the data point (x,y,v) at index i. If the subsequent point in the path (index $i+1$) has (x,y) coordinates within the Euclidean distance r, the point is appended to the segment and i is incremented by 1. The process continues until the subsequent point is outside of r. If the segment is equal to or longer than l it is returned and added to the path; if it is shorter than l it is discarded and a new segment is created at the point immediately following the previous one. l and r are parameters that I determined manually through trial and error to optimize performance. Though not shown here I experimented with performance improvements by varying l and r as a function of v, which may warrant integration into future data trimming protocols.

Once the entire path had been reconstructed from valid segments, a continuous temperature value (t) was assigned to each data point (*x,y,v,t*). *t* is calculated by linearly mapping *x* to the range of temperature values. The range of *x* is determined by taking the maximum and minimum values of the list of all *x* values (representing the leftmost and rightmost extremes the fish travelled to within the frame), and the range of temperature is determined from temperature ranges measured during long term tests of the RTPT-2 gradient (approx. 18.5-24.9°C).

This process iteratied through all fish IDs. The lists are concatenated into a Pandas (v2.1.1) dataframe with an additional column denoting fish ID. The dataframe is exported in .csv format for visualization and analysis in R.

2.6 Method evaluation & validation

Automatic tracking of multiple targets is inherently challenging (Butail & Paley 2010). Occlusion is the most common technical difficulty that researchers have encountered during the study of multiple individual fish in the same arena using optical sensors (Saberioon & Cisar 2016). A major downside of 2D tracking methods is that they fail to capture behaviour during occlusions, which includes situations where two targets overlap for several time units in succession, causing the temporary merging of those targets (Rodriguez et. al 2017). Investigators have developed several approaches to mitigate information loss during occlusions (see §1.4.1), but challenges still remain in tracking large groups of unmarked animals. The appearance-based identity-matching approach introduced by Pérez-Escudero et. al (2014) has shown the most efficacy in automatically detecting and correcting assignment errors, especially when combined with machine-learning augmentation. Prior to the current study this method has been restricted to groups of small individuals (<3cm in length) over short time scales. In the following sections I outline the results of applying this method to track goldfish (10-15cm) over longer timescales and suggest improvements and alternative approaches to study shoaling behaviour.

2.6.1 Tracking accuracy and group size

During the visual identification process, network predictions were evaluated using a heuristic termed uniqueness, which the network itself uses to estimate overall accuracy (or confidence) in identity assignment correction. Local (per-frame) uniqueness estimates local accuracy by assigning a confidence value for each measurement-target match, then evaluating overall confidence in that frame by rewarding high-confidence predictions and punishing low-confidence ones (Walter & Couzin 2021). Global uniqueness likewise estimates average accuracy by averaging local uniqueness from many evenly-spaced frames from throughout the video, and acts as a global gradient applied between each unit of training during SGD (see §1.4.4). Once training was complete, I used the final global uniqueness (with outputs that range from 0 to 1) as a heuristic estimating percentage accuracy in correcting assignment error compared to a perfect manual track.

Global uniqueness above 0.9 indicates high confidence in unique identity assignment, thus indicates minimal identity-switching and largely continuous, unsegmented trajectories. Although the training process is inherently stochastic, repeating the training typically generated similar uniqueness values (within < ±0.01), consistent with Walter & Couzin (2021).

Examining the effect of group size on tracking accuracy using global uniqueness heuristic as the dependent variable, I found that accuracy suffered dramatically as group size increased (Figure 2.6a). I acclimatized groups of untreated goldfish to the RTPT for a minimum of 1 hour, then recorded 30 minute videos of their unperturbed swimming behaviour. I tracked the fish groups in TRex and corrected identity assignment with a single round of visual identification training. I found that iterating the training process using the same training data provided minimal improvement to accuracy. The highest global uniqueness (0.93) was reported at the lowest population size of 4, and dropped off as population size increased, being the lowest (0.67) at the largest population size of 20.

I expected to see reduced identity assignment accuracy as group size increased, as occlusions increased in frequency, complexity, and duration as more individuals were added, but a significant drop-off in global uniqueness indicated a reduction in tracking quality due to assignment error propagation. Given the performance curve observed with the current protocol, I can still make general assessments about group properties but hesitate to draw solid conclusions at the individual level in group sizes larger than 4 due to a non-trivial likelihood of identities having not been conserved.

2.7 Summary

I designed, implemented, and validated a novel thermal enclosure that allows free thermoregulation and social interaction in a fish shoal. I developed a 2D multitracking protocol using the identity-based, machine-learning augmented software TRex, including a post-processing protocol for eliminating artifacts and producing continuous unique trajectories. This protocol achieves sub-par identification accuracy at larger group sizes but overcomes difficulties that previous studies have encountered when tracking larger objects for longer durations. Improvements to this protocol should include an analysis of how longer time-frames impact identity-based assignment correction, and incorporation of 3D techniques to resolve information about vertical position and movement. The data collected here provides the basis for an important conclusion in the application of tracking methods: the local accuracy of the tracking approach may be behaviourally dependent.

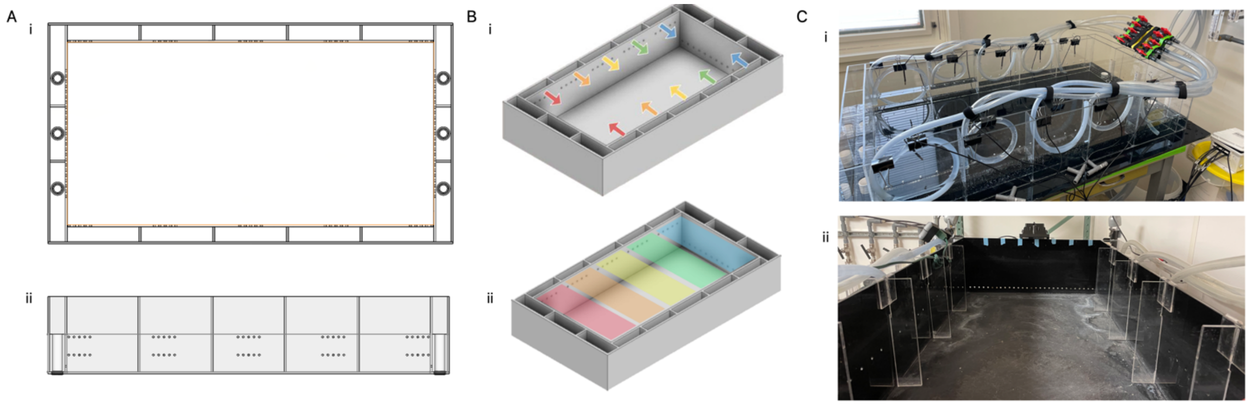

Figure 2.1. Rectangular Thermal Preference Tank (RTPT) functional schemata. A. i) Top-view schematic. Inflow channels are located top/bottom, and drainage sections are left/right. The entire inner rectangle forms the swimming arena. ii) Side view schematic. Pores are positioned equidistantly along the length of the arena in groups of 5. B. Isometric functional schematic. i) Arrows represent the location of input flow into the arena. ii) Coloured bars represent the theoretical temperature zones. C. i) RTPT Version 1 (RTPT-1) image demonstrating the water distribution system. ii) RTPT Version 2 (RTPT-2) image demonstrating an internal view as well as the 5cm baffles positioned on the border between the theoretical temperature zones.

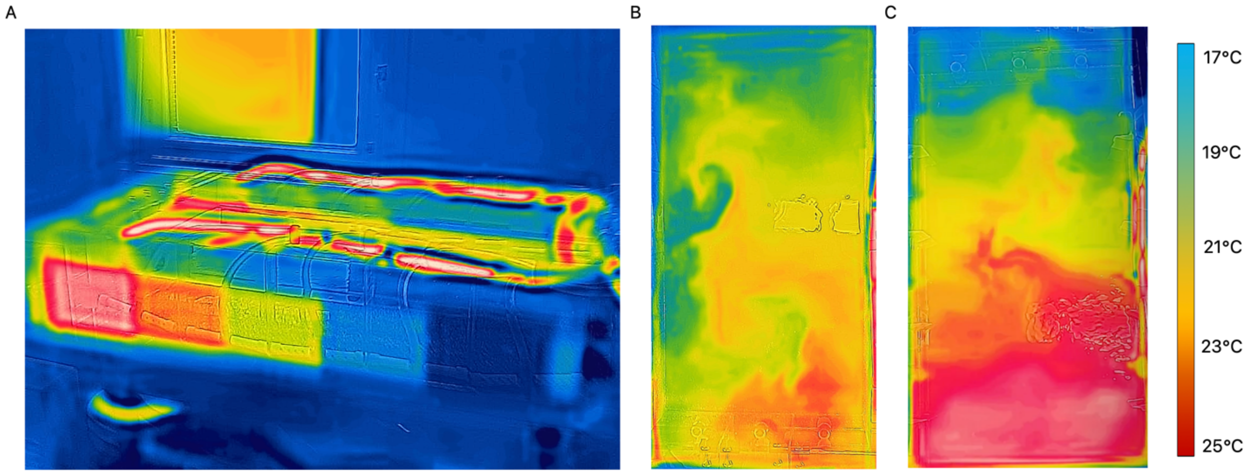

Figure 2.2. Thermal images of the Rectangular Thermal Preference Tank (RTPT). Images were captured with a FLIR ONE Gen3 mobile thermal camera attached to a Samsung mobile device. A. Side view of the RTPT-1 demonstrating temperature differential between inflow channels. B. RTPT-1 thermal gradient. RTPT-1 attained a maximum temperature differential of 5°C, while intermediate zone formation was hindered by circular flow patterns. C. RTPT-2 thermal gradient. RTPT-2 attained a maximum temperature differential of 6.4°C and formed more distinct thermal zones.

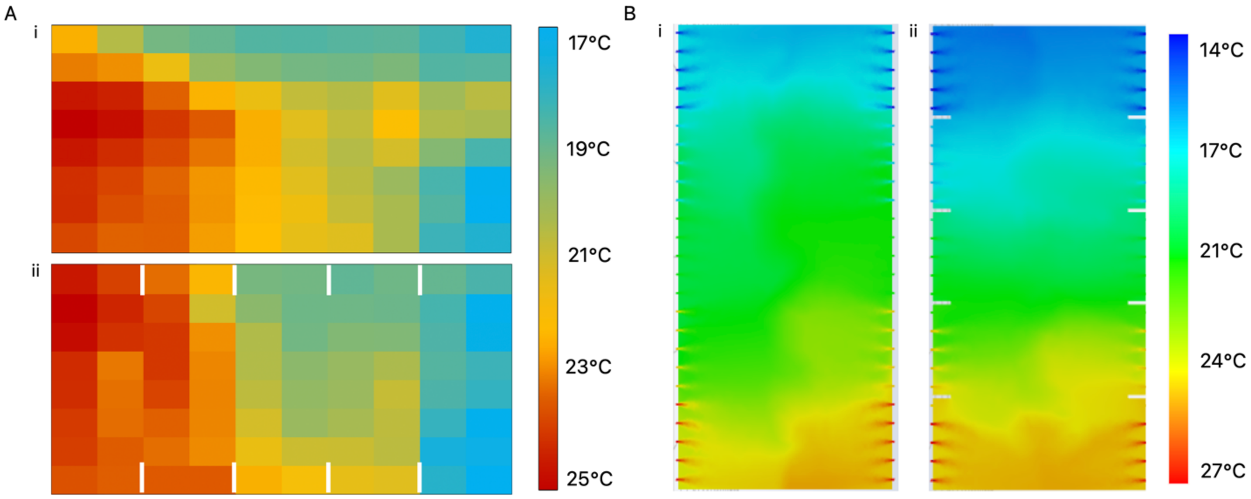

Figure 2.3. Baffles improve thermal zone distinction within the RTPT. A. Rectangluar Thermal Preference Tank (RTPT) thermal surface visualization without (i) and with (ii) 5 cm baffles attached bilaterally at the divisions between temperature zones (white lines indicate baffle location). Colour corresponds to 30 minute average temperature at the center of each grid cell at a depth of 2.5cm, captured on a per-minute basis using electronic thermocouples connected to a HOBOware U-30 data-logger. B. Computational fluid dynamics analysis without (i) and with (ii) baffles. Simulations were run in Autodesk CFD by collaborator Massiel Copara Chino.

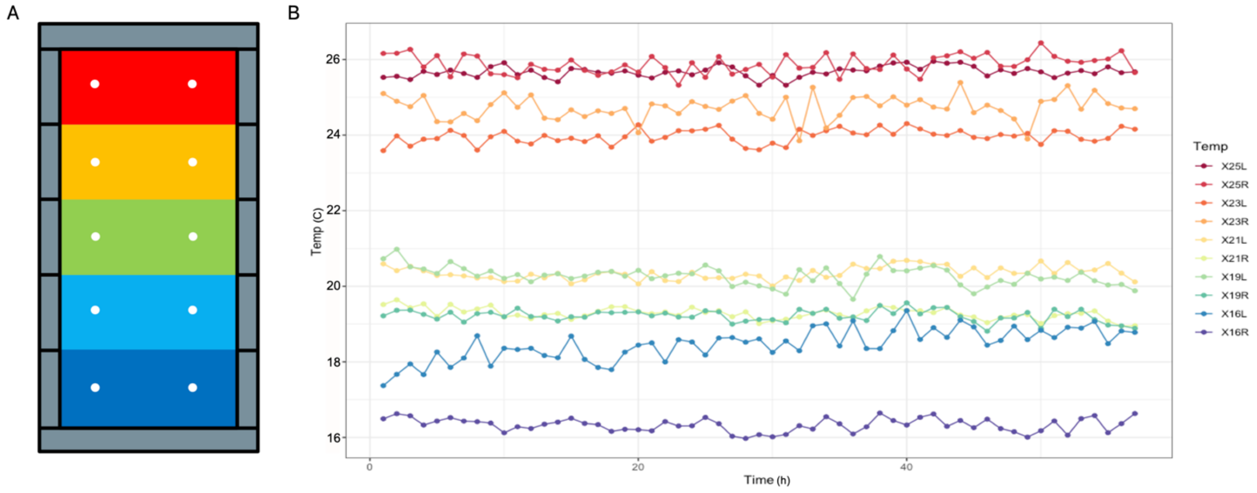

Figure 2.4. 48h RTPT thermal gradient stability trial. A. Rectangular Thermal Preference Tank (RTPT) schematic. White dots represent the positions of electronic thermocouples placed at 2.5cm depth. B. Average hourly temperature (°C) demonstrating thermal stability within each of the distinct thermal zones. Temperature was continuously recorded on a per-minute basis for a 48h period using 10 sensors connected to a Hoboware U-30 data-logger.

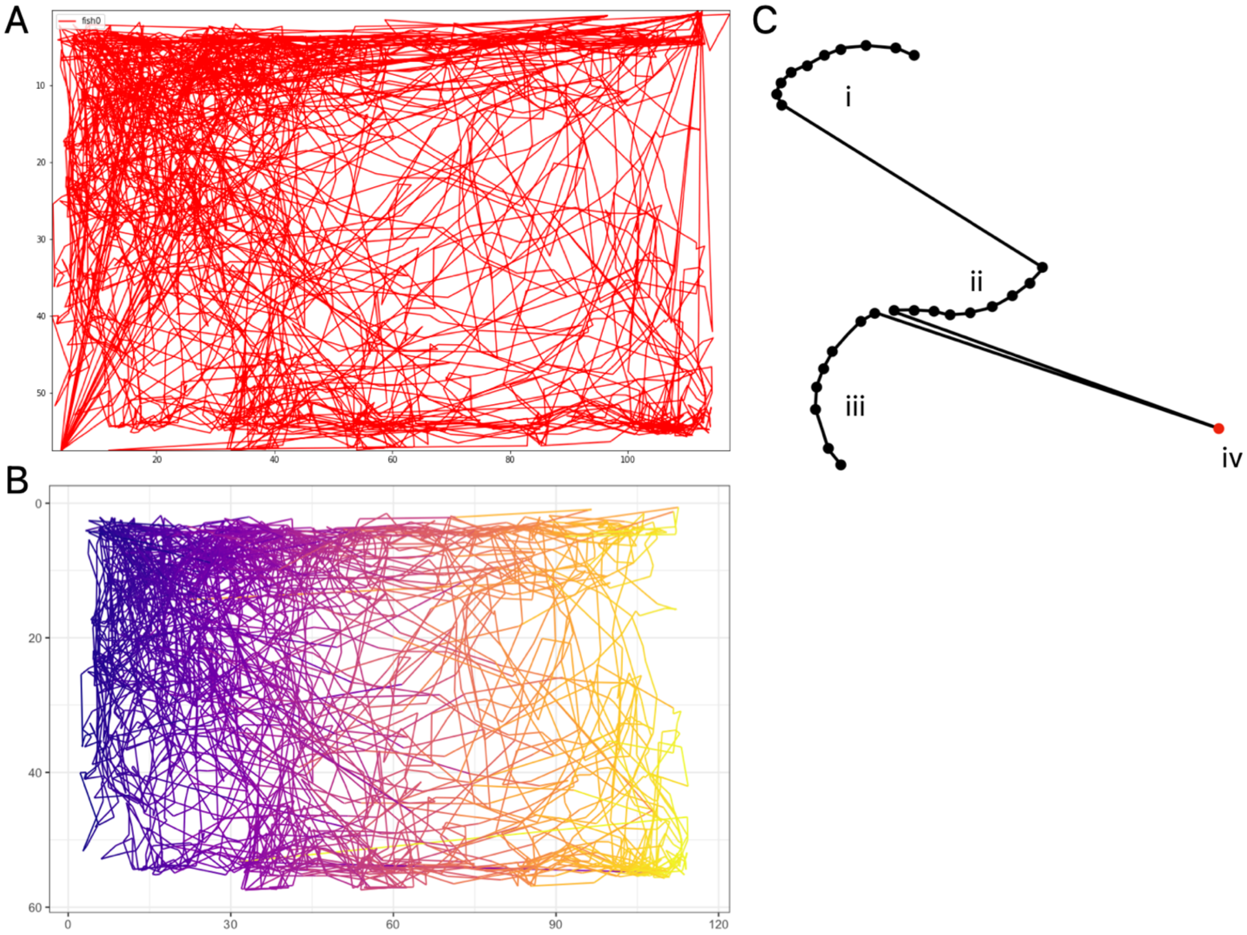

Figure 2.5. Post-processing in Python. A. An *x,y* plot of a representative fish path, containing true and erroneous data points. Plot generated in Spyder (v5.4.5) using the Python language (v3.11.6) and the matplotlib package (v3.8.0). B. The same fish path after post-processing. Tracking artifacts have been removed by reconstructing the path from valid, continuous segments. Colour represents temperature assignment based on the *x* coordinate. C. pathFilter3 demonstration. Segments i, ii, and iii are considered valid as they contain several continuous points. Point iv would be excluded, as it is not part of a valid segment. During post-processing, segments i and ii are joined despite not being continuous to account for data loss or identity switching.

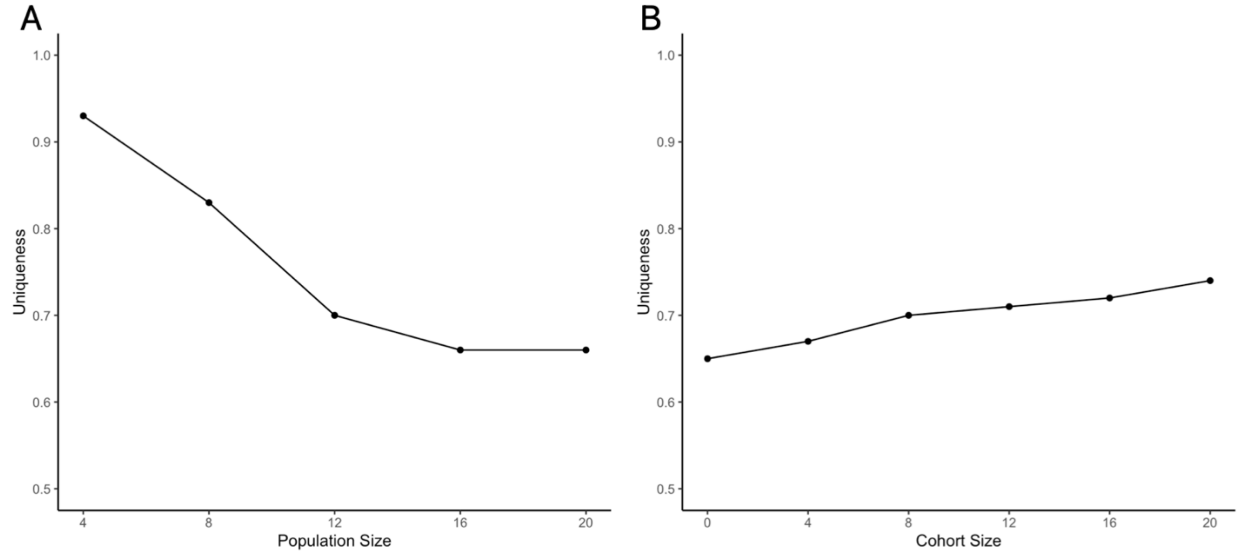

Figure 2.6. Group size and febrile cohort size influence visual identification performance. A. Groups of unmarked, untreated goldfish (*Carrasius auratus*) were acclimatized in the Rectangular Thermal Preference Tank (RTPT) for 1hr before 30min observation. Videos were tracked with TRex, which trains a neural network to identify individuals using appearance information. The global uniqueness heuristic is used here to estimate percent accuracy in identity assignment compared to a perfect manual track. B. Groups of 20 goldfish were tracked for 1 hour in the RTPT following 2 hour acclimatization. Prior to acclimatization, cohorts were injected intraperitoneally with 2.5mg zymosan, a fungal mimic which induces elevated temperature selection and lethargy (behavioural fever) 2-22 hours post injection. Videos were tracked as above, and global uniqueness is reported as a function of febrile cohort size.

Chapter 3: Results & Discussion

3.1 Abstract

Elucidating the links between shoaling behaviour and thermoregulation remains a challenge despite both being crucial to fitness in gregarious fish species. Behavioural thermoregulation is critical to maintaining an optimal temperature range for physiological rates, hatching success, growth, and development of offspring, while shoaling behaviour creates areas of safety that lower stress and benefit cooperative activities like foraging, predator defense, and reproduction.

How are group behavioural metrics influenced by shoal size, temperature preference, altered thermoregulation, and individual variation? In Chapter 2, the current study presented a protocol for multitracking fish schools, which captures the posture, position, and velocity of a shoal of up to 20 goldfish for 3 hours in per-frame resolution. I applied this method to carry out two proof-of-concept trials to examine the effects of group size and febrile presence on the behaviour of homogeneous and heterogeneous (febrile and afebrile) schools of goldfish. I found that larger shoals of goldfish preferred colder temperatures and swam slower than smaller shoals. Individual differences in speed were more repeatable than differences in temperature preference. The presence of a febrile cohort within the shoal increased variation in the temperature preference, but not speed, of the afebrile cohort in some groups. These proof-of-concept experiments inspire promising future directions becuase studies that use this protocol and apparatus can now address gaps regarding how shoaling behaviour, thermal preference, and individual variation are related in their contribution to fitness.

3.2 Introduction

Shoaling is a common behaviour among gregarious fish that confers a survival advantage to the group but is driven by contributions from many individuals exhibiting their own unique personality (Rey et. al 2015). Fish shoals appear egalitarian despite individuals engaging in the behaviour selfishly, electing to engage when the benefits outweigh the risks (Pitcher 1986). Shoaling provides some protection from predation and increases foraging success, but the mechanisms by which it does this are still unclear (Polyakov et. al 2022; Walsman et. al 2022).

Shoaling is challenging to study in ecologically relevant conditions. Field methods to track multiple individuals are expensive and suffer during inclement weather and environmental occlusion (Handegard et. al 2005; Polyakov et. al 2022). Laboratory studies are confounded by behavioural disruptions and biases created within experimental arenas (Bartolini et. al 2015; Macnaughton et. al 2018).

The current study sets the stage to address knowledge gaps regarding how the size of the shoal influences group-level metrics. Group size in shoaling fish varies widely in the wild and are challenging to predict; smaller shoals may move more cohesively and incur less intraspecies competition but are more vulnerable to unexpected attacks, while large shoals benefit from collective vigilance and cover more foraging ground but are more visible to predators (Magguran 1990; Pitcher et. al 1982; Pitcher 1986; Ward et. al 2011).

Thermal preference is a behavioural metric of particular interest in part due to its ecological relevance. Thermoregulation in fish is determined by the thermal preferendum, the behaviourally selected range of temperatures which optimizes physiological rates, fertility, growth, and maturity (Beitinger & Fitzpatrick 1979; Wallman & Bennett 2006). Thermal preference of freshwater fish ecologically defines their thermo-stable niche, while in marine species thermal preference may contribute to shifts in geographical range under ocean warming (Fisher et. al 2021; Perry et. al 2005). Thermoregulation also modulates immune function through processes like behavioural fever, discussed further in section §3.4. Elucidating the patterns and interactions that govern thermoregulation in social fish species may be critical to informing conservation strategies and tracking ecological shifts created by climate warming (Maldonado-Chaparro & Chaverri 2020).

The current study also aims to examine contributions of animal personality to shoaling behaviour by deconvolving individuals from the shoal. To a large extent it is still unknown how individual variation contributes to group-level metrics like group size, cohesion, and thermal preference, nor is it clear how group size influences survivability, reproductive success, and behavioural metrics.

This chapter presents two preliminary studies, the first of which examines the effect of group size on group-level temperature selection and speed, while examining individual contributions to behavioural variation. I investigated this relationship using the novel experimental arena and multitracking protocol presented in Chapter 2. The second study examines the behavioural outcomes on a population in which a cohort of individuals is expressing behavioural fever while the rest are unperturbed using temperature selection and speed as cohort-level metrics.

3.3 Group size influences group temperature preference and speed

Would larger shoals differ substantially from smaller ones in their overall temperature preference? A shift in temperature preference may underly the influence of shoal size on foraging success and predator risk (Gil & Hein 2017; Polyakov et. al 2022). If shoaling incurs metabolic savings from collective stress reduction, then larger shoals may select cooler temperatures overall (Pagnussat et. al 2013; Nadler et. al 2016). To examine whether increasing group size would result in a phenotypic-level cold shift in temperature preference, I acclimatized groups of untreated goldfish to a linear temperature gradient (18.5-24.9°C) in the RTPT-2 for at least 1 hour before recording their unperturbed swimming for 30 minutes. I tracked the behaviour according to the protocol described in Chapter 2 and analyzed temperature preference as a response variable in separate linear mixed models (LMM) for each group size using the lme4 package v.1.1-35.1 (Bates et. al 2015) in R Studio (v.2023.12.1+402). I found the most likely true average temperature value and 95% confidence interval for each group size (Fig 3.1a).

If larger shoals move differently than smaller ones, changes in kinetic properties such as average speed may underpin fitness benefits to certain group sizes. I hypothesized that a colder temperature shift in larger shoals would be accompanied with a decrease in overall speed. To test this hypothesis I repeated the above procedure using speed as the response variable (Fig 3.1b).

Both temperature preference and speed demonstrated a small but significant negative correlation with group size (Fig. 3.1c), indicating that the larger groups swam slower and preferred slightly lower temperatures overall. These data support the hypothesis that increasing group size creates negative shifts in speed and temperature preference, but further work is warranted to determine if these shifts inform optimized group size in wild populations.

3.4 Group-level metrics respond differently to individual variation

How does individual variation contribute to the downward shifts in temperature preference and speed observed as group size increases? Are repeatable individual differences in behaviour more pronounced at certain group sizes? I performed an individual-level analysis of the dataset above by constructing a global LMM with the dataset above using group size as a fixed factor. I partitioned the total behavioural variation into among-individual variance and residual (remainder) variance. Repeatability (*R*) was calculated as the ratio of among-individual variance to total variance.

Similarly to the independent models, the global LMM assigned a small negative slope (β) to the dependent variables of speed (-0.051) and temperature selection (-0.051) when plotted against the fixed effect of group size. The 95% confidence intervals around β in both cases were non-overlapping with 0 (Table 3.1). These data corroborate with the independent group size LMMs in indicating that adding individuals to the group created a negative shift in speed and a colder shift in temperature preference. Speed demonstrated a higher repeatability (*R*=0.394) than temperature (*R*=0.171), indicating that consistent individual variation in speed contributed more to overall behavioural variation than temperature preference (Table 3.1). Group size did not have a significant effect on among-individual variance, residual variance, or repeatability (Fig. 3.1a,b).

These data preliminarily indicate that individual variation did not significantly contribute to group-level behavioural shifts, but speed demonstrated more repeatable individual differences than temperature preference overall. Since more plastic behaviours (those that respond more readily to environmental change) are typically less repeatable (Bell et. al 2009; Nakayama et. al 2016), individual speed during shoaling seems less dependent on the number of neighbors nearby, and more dependent on individual preference. Conversely, temperature preference may be more plastic in that it is more easily influenced by social context. Further work must be done to determine how group dynamics shift at the group- and individual-levels as group size increases.

3.5 Behavioural fever in goldfish modulates the behaviour of afebrile shoal mates

Fever is a hallmark of acute inflammation, long recognized as a key indicator of host-pathogen interactions, and widely conserved for over 550 million years of metazoan evolution (Kluger et. al 1996). Recent work in this laboratory and others have renewed interest in the links between thermoregulation and fitness in fish (Gräns et. al 2012; Boltaña et. al 2013; Rey et. al 2017; Haddad et. al 2023). Endothermic and ectothermic fever are biochemically homologous, making fish a promising comparative model system to address fundamental knowledge gaps about the adaptive potential and mechanisms driving fever (Rakus et. al 2017). Illuminating these knowledge gaps could circumvent deadly outbreaks in aquatic facilities by informing the design of animal health practices that take advantage of natural thermoregulatory defenses (Huntingford et. al 2020).

Aquatic poikilotherms express behavioural fever upon infection in the form of hotter (2-4°C) temperature preference and lethargy as they lack the metabolic toolkit available to endotherms (Rakus et. al 2017). Despite the persistent medical paradigm regarding fever as a maladaptive symptom of disease (Bennett & Nicastri 1960; Kluger et. al 1996) a survival benefit attributable to mild fever has been established using ectothermic models and endothermic models (Kluger et. al 1975; Satinoff et. al 1976; Husseini et. al 1982). Our lab mechanistically investigated this survival benefit in goldfish, finding that behavioural fever more effectively mounted innate defenses against infection with a pathogen by selectively altering leukocyte recruitment, promoting more efficient control of inflammation, and accelerating tissue repair (Soliman et. al 2023; Haddad et. al 2023).

Could an adaptive social benefit also contribute to the persistence of fever in vertebrate clades? Shoals and schools react to behavioural stimuli in concert due to effective communication of visual and chemical signals (Magurran & Higham 1988l; Ward et. al 2011). If behavioural fever is such a signal, would we see a behavioural change in healthy shoalm ates as the number of febrile ones grows? To see if altering the number of febrile fish in the group produced a behavioural change in the healthy cohort, I tracked six groups of 20 goldfish that contained different proportions of afebrile to febrile fish (afebrile:febrile; 20:0, 16:4, 12:8, 8:12, 4:16, 0:20). Fish were herein considered febrile when they received a treatement which has been shown to consistently induce a self-resolving fever response in closely related established models. The febrile treatment was 25mg (100µL) intraperitoneal zymosan injection whereas fish in the afebrile treatment received no injection. I observed the groups during the febrile window the same day (at least 2 hours following injection). I acclimatized the fish to the RTPT for at least

1 hour before recording their behaviour for 2 hours. I tracked the videos with TRex following the protocol described above and found the mean temperature and speed preferred by each individual over the 2 hour period. Figure 3.1a shows the average temperature (Figure 3.1b; speed) occupied by each cohort, afebrile and febrile, for each of the six groups, found by taking the mean of the individuals among each cohort.

Consistent with previous findings, the fully febrile group (0|20; this notation refers to afebrile|febrile) had a mean temperature 1.9C above that of the control group (20|0) (Fig. 3.1a) and demonstrated the associated lethargy response with a mean speed 0.89cm/s slower than the control group (Fig. 3.1b). In the mixed groups, febrile fish did not display markedly different behaviour in the presence of afebrile conspecifics, while in the 12|8 and 8|12 groups afebrile fish exhibited more variance in their temperature selection (Fig. 3.1). In other treatments, afebrile behaviour tended to approach that of the febrile individuals.

These preliminary data indicate that behaviour is less plastic during fever, as the thermal preference and lethargy were not disrupted by the presence of healthy conspecifics. Although the relationship is not clear, there is a trend indicating that healthy individuals in mixed groups with a significant but not predominant febrile cohort were more variable in their temperature preference. The increase in variance supports the finding that temperature was less repeatable and dependent on social context. Increased variance in temperature preference may represent disparate behavioural responses to the febrile cohort; those that choose to remain close to the slower moving febrile group, and those that avoid it. An increase in behavioural variation could also have arisen from a stress response induced by the presence of sick individuals. There is indication of group-level changes in response to fever, supporting further investigation of the hypothesis that fever is a social signal that promotes beneficial behavioural shifts.

3.6 Discussion

3.6.1 Phenotypic-level metrics varied with group size in healthy shoals

Upon tracking different sizes of goldfish shoals for 30 minutes I found that larger shoals swam slower and preferred colder water on average. Limited space within the arena may explain the decrease in speed with increasing group size. Larger shoals took up more space in the arena which restricted unencumbered swimming. I anecdotally observed that goldfish in this study generally tended to slow down and physically contact while crossing over each other, meaning an increased number of interactions might contribute to an overall reduction in speed. However, this observation has yet to be quantified.

Regarding the fishes' shifts to cooler temperature preference when experiencing larger group sizes, larger shoals may have experienced anaerobic stress resulting in selection of colder, more oxygen rich water (Domenici et. al 2016). Larger shoals may have instead experienced a reduction in stress, causing short-term metabolic shifts resulting in a slightly decreased temperature preference (Nadler et. al 2016). Future trials including more shoal sizes may reveal a negative curvilinear temperature selection, inflecting at the point where these benefits are outweighed by crowding.

Large shoals in the wild provide protection but cover more volume and are visible from further away (Rieucau et. al 2015). Large shoals may risk selecting warmer water where predators have an advantage to access valuable foraging patches. However, this may only happen in the context of a predator threat, as the presence of a predator has shown to impact thermal preference of fish significantly (Magurran & Higham 1988; Kuruvilla et. al 2022). Individuals within a large shoal may express their temperature preference over the entire shoal volume while remaining a member, though I did not observe any effect of group size on the repeatability of temperature preference.

3.6.2 Shoal size affected speed more consistently than temperature preference

I found that 39.4% of total behavioural variation in speed across all group sizes was attributable to repeatable individual differences (Table 3.1). If more repeatable traits tend to be less plastic (Nakayama et. al 2016) then the data may indicate that speed is less plastic with regards to social context (number and behaviour of neighbours). Repeatable individual differences in temperature preference made up 17.1% in comparison, indicating that individuals did not exhibit distinct preferences within the group that contributed to overall behavioural variation.

Though the relationship is not clear, group size induced opposite trends in the residual variance of speed and temperature preference. Through repeated trials one could partition within-individual variance from the residual, though it is likely that within-individual variance (how consistently individuals behave over time) makes up a large part of the residual variance (Bell et. al 2009; Bierbach et. al 2017). Under this assumption, if within-individual variance in speed decreased with group size individuals were more likely to vary their preferred speed in larger shoals (Fig. 3.1b). In comparison, individual temperature preference seemed more consistent in larger shoals (Fig 3.1a). Group size had no significant relationship towards individual temperature selection or speed, although in the smallest group size (n=4) among-individual variance made up 53.2% of the variance in temperature preference, indicating individuals selected temperatures both consistently and distinctly (Fig. 3.1a).

Further repetitions of these experiments are required before conclusions can be drawn from these data regarding the relationship between group size, among-individual variation, and behavioural repeatability. However, the largest shoals tended to have the lowest among-individual variation, while the smallest had the highest among-individual variation. This could be an artifact caused by decreased tracking accuracy, as data from the larger shoals likely contained assignment errors that reduced the uniqueness of each trajectory.

3.6.3 Behavioural fever is a social signal shifting group responses to disease

Frequently associated with host-pathogen interactions, fever is conserved throughout vertebrate taxa and displays strong evidence of positive selection in association with temperature-dependent innate defenses (Kluger et. al 1996; Rakus et. al 2017). Furthermore, positive selection towards pathogenic elements taking advantage of fever is clear in our co-evolved pathogens (Casadevall 2016). However, there is a longstanding debate over the adaptive benefit of fever and its metabolic costs (Hasday et. al 2000; Evans et. al 2015). Recent studies have begun to uncover the mechanistic basis behind the survival benefit attributed to fever and how it integrates with innate immune defense at multiple levels (Gräns et. al 2012; Boltaña et. al 2013; Soliman et. al 2023; Haddad et. al 2023).

From a social behaviour perspective, the behavioural fever model in fish is well-suited to study behavioural dynamics within groups of fish expressing distinct behavioural programs. The model is similarly suited to examine the consequences of shifting the spatial arrangement of shoal members through altered thermoregulation. Recent results from this laboratory indicate that fever boosts innate defense through efficient inflammation control and tissue repair, leading to greater wound healing and reduced pathogen shedding. Could fever disrupt the transmission of disease within a shoal by reducing pathogen shedding and altering the spatial arrangement of shoal members?

The current study sets the stage to answer this question by overcoming methodological challenges and by looking at shifts in group dynamics created by behavioural fever by deconvolving febrile and afebrile cohorts within the same shoal. I found that healthy individuals in mixed groups of 20 fish that contained a cohort of at least 8 febrile fish were more variable in their temperature preference, but not speed (Fig. 3.2). Similarly, speed in healthy shoals was more repeatable and less plastic than temperature preference with regards to nearby conspecifics (Table 3.1). Further, if temperature preference were to be confirmed as being less repeatable than speed, then the data align with the conclusion that temperature selection is more socially plastic (more sensitive to variation in social context) than speed.

Febrile individuals varied little in the mean and variance between groups, indicating that the temperature preference and speed of febrile fish were more invariant to conspecific activity. These data preliminarily suggest that febrile fish are resistant to deviation from their preferred temperature while modulating the behaviour of nearby conspecifics. There is evidence that shoaling facilitates communication of social signals, and further investigation is warranted into the possibility that behavioural fever functions as such a signal. If behavioural fever prompts behavioural changes in healthy shoal mates, then might behavioural fever offer a protect against transmission of disease by reducing individual infectivity and promoting pro-tolerance shoal arrangements? If so, then behavioural fever and shoaling may be co-selected adaptive traits that provide a fitness benefit in concert through the exertion of individual variation in thermal preference.

3.7 Summary

These proof-of-concept findings highlight the capabilities of the method described in Chapter 2 and begin to address the links between thermoregulation, shoaling, and immunological defense. Group size had a significant effect on the behaviour of goldfish shoals with respect to both temperature preference and speed, demonstrating that group size modulates group metrics crucial to survival. Febrile behaviour was largely unaffected by the presence of healthy shoal mates, whereas healthy fish changed their behaviour in response to the presence of a significant febrile cohort. These preliminary findings warrant further investigation into fever’s role in disease transmission within gregarious fish shoals.

Repeatable individual differences did not significantly contribute to shifts in temperature preference or speed, but speed was a more repeatable metric than temperature preference in this study. The contributions of individual personality to group behaviour remains to be further studied. These findings build upon previous studies and lend new insight into shoaling behaviour within homogeneous and heterogeneous groups.

Table 3.1 Result from global linear mixed model (LMM) using temperature and speed as dependent variables. The global model is of the form X~Group Size+(1|GroupID), where X is the dependent variable, GroupID is the grouping factor, and Group Size is a fixed factor. GroupID is a composite variable that uniquely identifies each fish and its group. Total behavioural variation is partitioned into that dependent on GroupID (among-individual variance) and the remainder (residual variance). Behavioural repeatability is found as the ratio of among-individual variance to total variance. Models were generated using the lme4 package v.1.1-35.1 in R Studio v.2023.12.1+402.

Dependent Variable	Temperature	Speed
Fixed Effects	β (95% CI)	
Intercept	23.579 (22.943,23.922)	6.766 (5.711,7.271)
Group Size	-0.047 (-0.069,-0.006)	-0.051 (-0.100,-0.006)
Random Effects	σ (95% CI)	
Among-individual	0.354 (0.313,0.477)	1.059 (0.917,1.168)
Residual	2.008 (1.862,2.152)	1.589 (1.486,1.714)
Repeatability	0.171 (0.136,0.196)	0.394 (0.353,0.429)

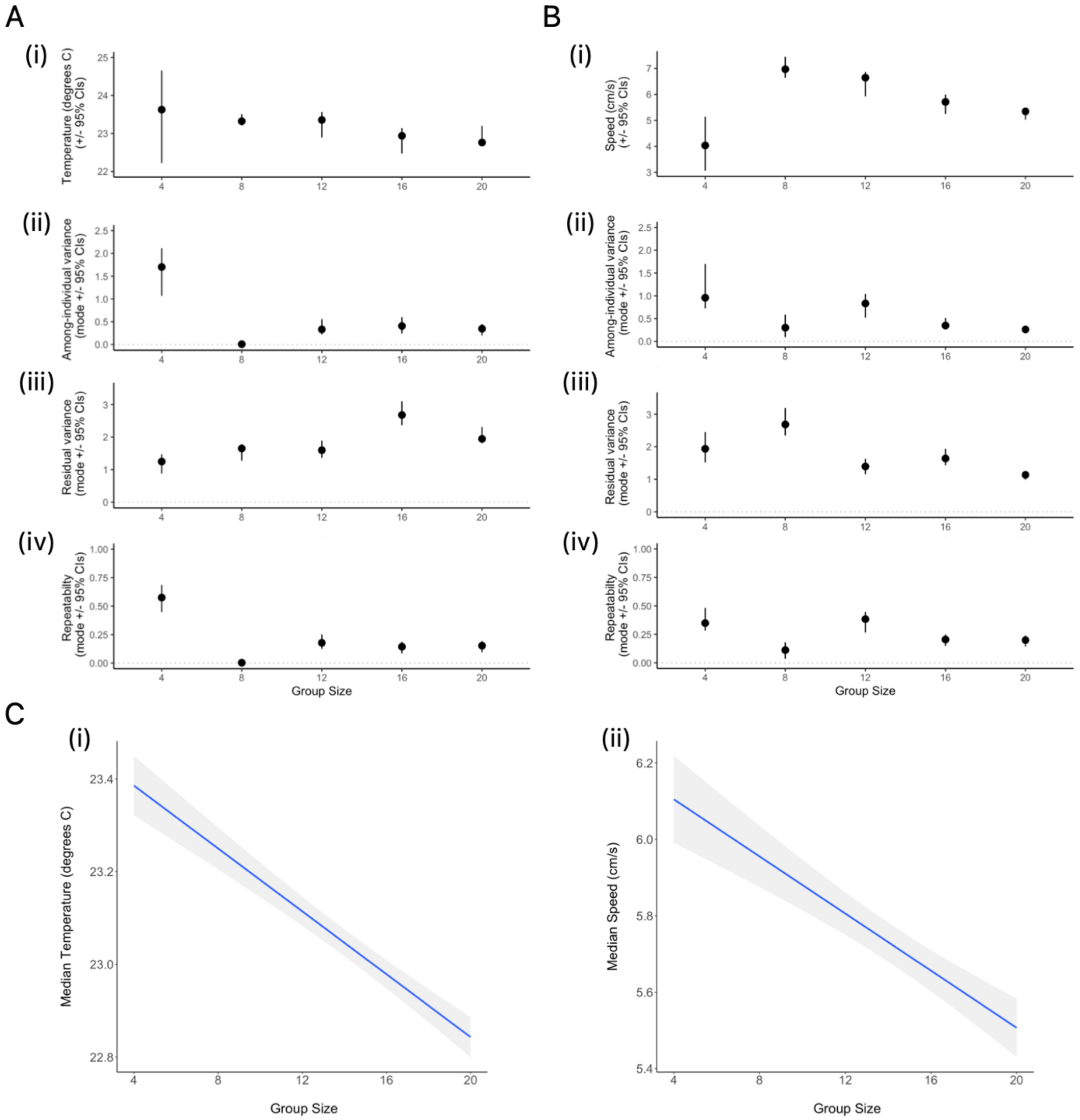

Figure 3.1 Larger goldfish groups prefer colder temperatures and swim slower. A. Temperature (°C) and B. speed (cm/s) were analyzed as response variables in separate LMMs using the lme4 package v.1.1-35.1 in R Studio (v.2023.12.1+402). The models were of the form *X*~1+(1|FishID), where *X* is the response variable and FishID is the grouping factor. Total behavioural variation was partitioned into ii. variance dependent on GroupID (among-individual variance) and iii. the remainder (residual variance). iv. Behavioural repeatability was found as

the ratio of among-individual variance to total variance. C. Regression plot between group size and i. Temperature (°C) and ii. Speed (cm/s) using a global LMM (see Table 3.1).

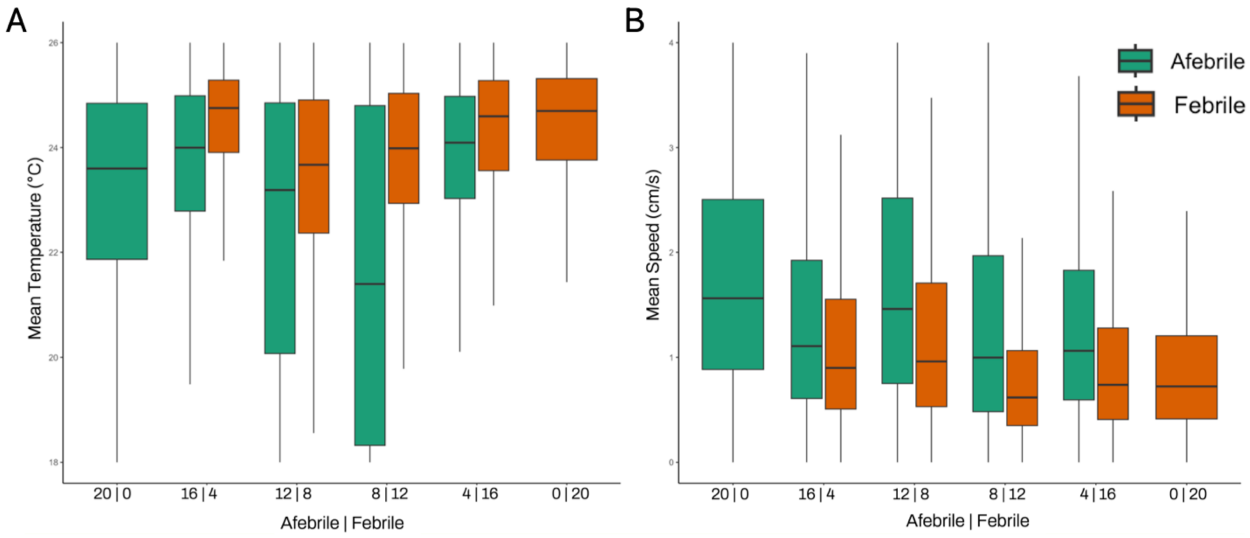

Figure 3.2. Febrile cohorts modulate behaviour of afebrile conspecifics. Goldfish reared at 16°C were acclimatized to a linear 18.5-24.9°C gradient in the RTPT for at least 1 hour before behavioural observation for 2 hours. Fish in the afebrile treatment were uninjected and fish in the febrile treatment were injected intraperitoneally with 25mg zymosan and observed during the febrile peak (at least 2 hours post injection). Mixed groups were formed using different proportions of afebrile and febrile fish, indicated on the x axis. Video observations were analyzed with TRex. The center of each boxplot represents A. mean temperature (°C) and B. mean speed (cm/s) of each cohort, found by sampling the median temperature and speed per minute for each individual.

Chapter 4: Conclusions & Future Directions

4.1 Introduction

There are a growing number of machine vision tools promoting investigation into previously interminable questions about kinetics, structure, formation and development of social behaviour. Contributions of individual personality to social behaviour have been particularly challenging to resolve due to the complexity of capturing group behaviour while perfectly maintaining unique identities throughout the observational period (Viscido et. al 2004). Accumulating evidence from a wide variety of species support the paradigm of animal personalities which suggests that some individuals are consistently more aggressive, more exploratory, or more bold than other individuals and that these consistent individual differences in behaviour are often heritable and related to fitness (Biro et. al 2010; Rey et. al 2015).

Shoaling and schooling in fish provide measurable benefits to collective vigilance, foraging, and communication (Pitcher et. al 1982; Magurran & Higham 1987; Ogino et. al 2023). Contrary to hierarchical social groups, fish shoals and schools respond to stimuli through egalitarian contributions of many individuals electively engaging in the group (Pitcher 1986; Drummond 2006). To open new avenues of investigation into the links between shoaling, anthropogenic thermal stress, disease transmission, and fever, the current study focused on a methodological approach to resolving among-individual variation during shoaling.

Quantifying environmental preferences within the laboratory requires specialized experimental aquaria. Researchers studying temperature preference have chosen aquaria that limit selection to discreet chambers or otherwise disrupt the natural movement of fish within the apparatus (Myrick et. al 2004; Kuruvilla et. al 2022). With the aim of studying shoaling behaviour under free thermoregulation, I established and validated an aquarium which addresses these limitations while accommodating continuous observation of shoals within an open arena.

Chapter 4 discusses potential applications of the RTPT apparatus and further improvements worth considering. I also discuss the challenges I encountered when applying a machine-learning appearance-based approach to multitracking a shoal of goldfish. I suggest alternative approaches to capturing unique individual information during group behaviour.

4.2 Tank improvements

The RTPT-2 continuously maintained a stable 6.4°C thermal gradient (18.5-24.9°C) for a period of 48h. I chose that temperature range as it was previously observed that goldfish occupy temperatures as warm as 26°C during the febrile period but 24.9°C was the warmest temperature that could be maintained by mixing the facility's hot (26°C) and cold (9°C) water sources. The temperature range and maximum differential can be finely adjusted within the limitations of the water source temperature. In future studies it would be beneficial to increase the temperature differential to include both the full febrile range and habituated temperature of the goldfish (16-26°C).

Due to the exceptional long-term stability of the thermal gradient in the RTPT-2, it would also be possible to more precisely estimate the temperature of a target in the arena. The current protocol assigns temperature using a linear map from the range of *x* coordinates to the range of temperature (Appendix 4.1a), which approximates a temperature gradient that is linear in the *x* direction and invariant in the *y* direction. Temperature data could be used to calculate a function that more closely maps the temperature shift (Appendix 4.1b), or calculate such a function for each *y* value, generating a family of functions that approximates the (*x,y*) temperature surface, from which a precise temperature can be sampled (Appendix 4.1c). Depth information should also be added to the approximation, since temperature changes with depth even in a shallow arena. Future tank versions will be adjusted to limit behavioural biases, for example rounding corners to discourage limit edge effects where fish either avoid or hide in corners.

4.3 Factors inhibiting tracking performance

4.3.1 Group size

Upon tracking goldfish shoals of different sizes using the method described in Chapter 2, I found that accuracy in identity assignment decreased as group size increased (Fig. 2.6a), indicating that the method was successful in tracking small groups but required a secondary form of identity verification either in the form of tags or manual correction. Future approaches should consider using a combination of machine-learning tools and non-invasive tags such as coloured bands, beads, or fluorescent fin marks to ensure identities are maintained even if the tags are obscured or identity-matching fails.

4.3.2 Analysis duration

The current study did not properly establish the effect of analysis duration on tracking accuracy, but longer videos tend to accumulate errors over time which makes perfectly maintaining identities increasingly difficult. Walter & Couzin (2021) tracked 76 minute and 3 hour videos of eight guppies and reported global uniqueness of 0.96 and 0.91 respectively, indicating that the length of the video had a marginal negative impact on global uniqueness. The remainder of videos analyzed in TRex reported higher global uniqueness, but none were longer than 10 minutes. Other authors that achieved success using similar recognition techniques to track fish shoals (Qian et. al 2016; Wang et. al 2017; Romero-Ferrero et. al 2019) typically limit their analysis to short videos, indicating that this approach is perhaps best applied to solving many occlusions within a short time frame. Other approaches may be more suited to the challenge of perfectly maintaining identities during a long-term analytical study.

4.3.3 Species-dependent behavioural patterns

I found that behavioural tendencies in a shoal influenced global tracking accuracy, supporting the hypothesis that behavioural patterns cause changes to local accuracy. Shoals expressing fever where individuals were less likely to interact with each other demonstrated higher global tracking accuracy (Fig. 2.6b). Visual identification performs best when individuals are entirely in view and visually distinct from each other. Even when local accuracy is generally very high, identity assignment often fails when many individuals are in close proximity or visually overlapping.

Future studies should consider species-specific properties and behavioural tendencies when choosing a tracking approach. TRex has been successful in distinguishing individuals in short videos of groups up to 100 zebrafish and 59 fruit flies (*Drosophila melanogaster*). Other studies investigating shoaling behaviour chose guppies, giant danios, sunbleaks (*Leucaspius delinateus*), or neon tetras (*Hyphessobrycon herbertaxelrodi*), all typically under 3cm in length. These small organisms change little in appearance when viewed from above and move quickly compared to their body size. It is relatively simple to solve crossings between independent trajectories that last only a few frames. Existing tracking examples using this software lack examples of complex shoaling behaviour, zebrafish spending a majority of the time independently exploring the arena rather in close physical contact (Walter & Couzin 2021). Goldfish, which are 10-15cm long, can vary more from frame to frame than zebrafish as they are more deformable while swimming which creates more frame-by-frame variation in posture and body illumination. Targets that vary more in appearance may require a higher volume of training information to reliably match their images (LeCun et. al 2015).

In this study goldfish tended to swim much slower than zebrafish compared to their body size and spent more time swimming in close proximity to shoal mates. Goldfish crossing paths were more likely to slow down, physically overlap, and change trajectory entirely. The longer and more complex the occlusion, the lower the probability of successfully re-identifying the individuals after the event (Dolado et. al 2015). More variation in appearance requires much more training data for a neural-network to successfully recognize the same target in a myriad of visual states (LeCun et. al 2015). It would be instructive to use this method to compare tracking accuracy between groups of goldfish, smaller species (eg. zebrafish), and markedly less gregarious species.

4.5 Alternative tracking approaches

Automatic tracking of multiple targets is inherently challenging (Butail & Paley 2010) but challenges may be ameliorated if the chosen approach is suited for the desired application. For example, a tracking approach in which accuracy is dependent on appearance and gregariousness would be ill-suited for long-term behavioural studies where perfectly maintaining individual identities is a requirement. If local accuracy changes with the behavioural context in the scene, systematic biases and sampling error could occur when drawing conclusions at the individual level.

4.5.1 Two-Dimensional approaches

A major downside of 2D identity-based tracking is that it fails to capture behaviour during occlusions, where two targets appear superimposed. Several image processing approaches have been proposed that use an erosion-dilation method, where the image of the occlusion is shrunk and expanded to resolve the individual contributions of each individual to the image (Kato et. al 2004; Dolado et. al 2015). However, these algorithmic approaches break down when solving more complex occlusions. Baum et. al (2013) proposed a two-layer method which uses a hybrid Kalman filter and game-theory related reinforcement. This method tolerates much longer occlusion times but has not yet been applied to tracking groups of animals. GroupTracker (Fukunaga et. al 2015) applies an expectation-maximization scheme to a Gaussian mixture model for distinguishing severely occluded targets. This method saw comparable success to idTracker (Pérez-Escudero et. al 2014) in tracking fish groups, was released around the same time, and could be enhanced with contemporary machine-learning approaches.

4.5.2 Three-Dimensional approaches

Studies about trajectory are mostly based on tracking in 2D space, but organisms live in three-dimensional (3D) environments, which extensively influences ecological interactions (Saberioon & Cisar 2016). For instance, it is difficult to recognize behaviour that includes vertical movement using 2D tracking. As such, tracking animals in 3D is more desirable in animal behavioural studies, despite being more challenging and computationally intensive. Occlusion is a common problem when tracking shoals in 2D, but incorporating 3D information would more easily allow resolution of individuals that are above or below each other. For example, Pautsina et. al (2015) constructed 3D images based on intensity of reflection of near-infrared radiation from the targets, which helps infer depth information about animal positions. A technique similar to digital particle image velocimetry may be used, creating rapid light flashes with variation the vertical plane to reveal 3D information (Nauen & Lauder 2002). Saberioon & Cisar (2016) tracked four 7cm long Nile tilapia (*Oreochromis niloticus*) using a commercially available single point structured-light sensor.

Stereo-cinematography techniques with mirrors (Zhu & Weng 2007; Maaswinkel et. al 2013; Kanbara et. al 2006) or multiple cameras (Matsumoto et. al 2013) have also been used to capture two simultaneous images from different angular positions, allowing resolution of 3D information. Difficulties with these systems come from automatically matching 3D stereo-point pairs and synchronizing multiple cameras, both of which require sophisticated calibration procedures and software (Stefano et. al 2004; Delcourt et. al 2013).

4.6 Conclusion

This thesis focused on a methodological approach to parse out individual contributions to shoaling behaviour. The rationale for this development was to open new avenues of investigation into fitness advantages underpinning optimum group size and thermal preference in gregarious fish. The contributions of individual personality to among-individual variance in behaviour underlie relevant questions in the fields of developmental biology, immunology, epidemiology, and climate ecology. How individualized behavioural reactions to stimuli sum to group-level behavioural phenomena is of particular interest.

4.6.1 Appearance-based identification performed worse during aggregated shoaling

The 2D appearance-based multitracking approach I used was successful in uniquely tracking small shoals of goldfish over long periods, but maintaining uniqueness was increasingly difficult as group size increased. Future studies examining the behaviour of 3D shoals should incorporate depth information to more easily resolve top-down occlusions. The approach presented here was prone to local shifts in tracking salience during aggregative shoal behaviour. The results demonstrate the complexity that arises when tracking larger, more deformable targets that are often in close proximity. Species-specific behavioural patterns may arise that require either more information to be gathered during tracking (eg. high quality training data, additional cameras, physical markers) or a designed approach that incorporates information about the species' behavioural tendencies.

4.6.2 Larger schools swam slower and preferred colder water

Using TRex to analyze freely thermoregulating shoals in the RTPT-2, I found that group-level temperature preference and speed both negatively interacted with group size. Speed was more repeatable, and perhaps less socially plastic, than temperature preference. Larger shoals tended to have less consistent individual speeds, but more consistent temperature preference. These preliminary results highlight differences in how behavioural traits respond to variation in group size, despite experiencing similar group-level shifts.

4.6.3 Febrile cohorts prompted behavioural shifts in afebrile conspecifics

Behavioural fever in fish demonstrates a survival benefit against infection by enhancing innate defence mechanisms. I found that goldfish shoals made up of 40-60% febrile fish experienced an increased variation in temperature preference, indicating that behavioural fever induced a behavioural response in healthy conspecifics. These findings warrant further investigation into the links between thermoregulation and shoaling, as shoaling may synergistically facilitate the transfer of fever as social signal promoting disease tolerance.

4.6.4 Relevance & applications

The methodological approach presented in this thesis could represent the basis for a computer-controlled system which automates animal health within an aquaculture enclosure. A machine-vision system and finely-controlled temperature gradient could automatically segregate susceptible individuals from outbreaks before any physical symptoms appear, or assist in identifying susceptible individuals for vaccination. I experimented with markerless appearance-based identification in poultry, which could be combined with behavioural deep-learning recognition to identify sickness behaviours such as aggression, fatigue, and abnormal sleeping patterns.

The data presented here suggest that the success of machine learning approaches requires mindful attention to the behavioural tendencies and visual dimensions of the species at hand. Currently, software claiming to be species-agnostic, like TRex, still have a long way to go in meeting tracking demands of organisms with multiple self-occlusions and shadows created by their body structure. However, tailored approaches like Marigold, a web-based zebrafish behaviour analysis platform, have recently achieved success with carefully constructed layer-structure within the network that reduces training times while retaining recognition salience. Teicher et. al (2024) used Marigold to examine the kinetic profile of head and tail stimulated hyperactivity responses, distinguishing glutamate transporter *slc1a2a* (Eaat2a) mutant *slc1a2b*$^{tk57/tk57}$ embryos from wild-type embryos.

Overall, this research preliminarily addressed and quantified the links between group size, thermal preference, behavioural fever, and individual variation in shoals of goldfish. I achieved this using an established behavioural fever model, markerless multitracking protocol, and novel thermal apparatus.

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Appendices

Appendix 2.1: Commented excerpt from the TRex post-processing protocol showing the pathFilter3 function.

The full code is found at https://github.com/eoprocto/trex_postprocessing

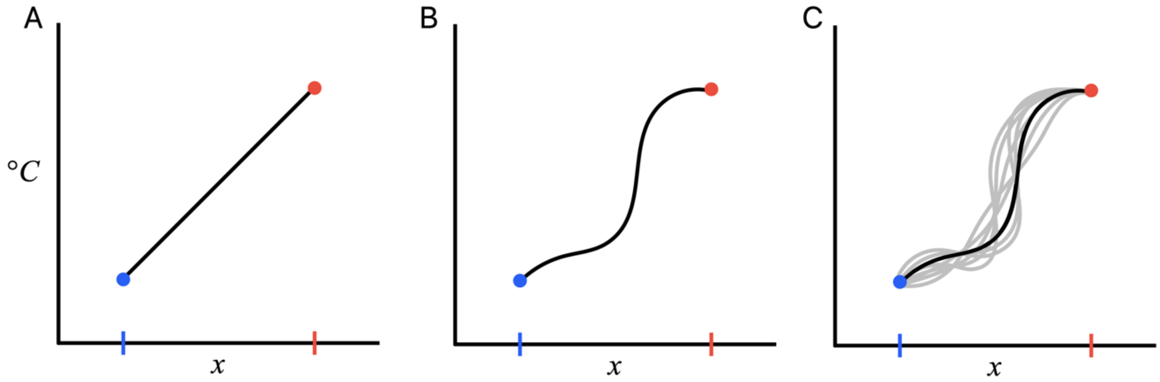
```
def pathFilter3(a,b,c,d):
    #a,b,c are the x,y,speed lists
    #d is fishID

    #create empty lists for reconstructed path
    A = []
    B = []
    C = []
    i=0
    l = 20
    #iterate through entire length of a (a,b,c have same length)
    while i<len(a)-l:
        #createSegment returns continuous path segment starting at i
        A_seg,B_seg,C_seg = createSegment(a,b,c,i)
        #segment is added to path if long enough
        if len(A_seg)>=l:
            A = A + A_seg
            B = B + B_seg
            C = C + C_seg
        i=i+len(A_seg)+1
    print('fish '+str(d)+'|','original: '+str(len(a)),'new: '+str(len(A)),'removed: '+str((len(a)-len(A))))
    return A,B,C

def createSegment(a,b,c,i):
    #a,b,c are x,y,speed lists

    #initialize segment with data point at i
    x,y,z=a[i],b[i],c[i]
    x2,y2=a[i+1],b[i+1]
    A_seg=[x]
    B_seg=[y]
    C_seg=[z]
    #r defines maximum distance between two points while still being continuous
    r=10
    #extend segment while subsequent points are within r of each other
    while calcDistance(x,y,x2,y2) < r and i<len(a)-2:
        i=i+1
        x,y,z=a[i],b[i],c[i]
        x2,y2=a[i+1],b[i+1]
        A_seg.append(x)
        B_seg.append(y)
        C_seg.append(z)
    #Once the next point is outside of r, return the segment
    return A_seg,B_seg,C_seg

#returns Euclidean distance between (x,y) and (x2,y2)
def calcDistance(x,y,x2,y2):
    return sqrt((x-x2)**2 + (y-y2)**2)
```



Appendix 4.1. Example of improved temperature determination in a semi-linear thermal gradient.

A. The current protocol simply uses a linear transformation between the range of *x* the range of temperature, assuming the coldest temperature occurs at the leftmost *x* value and the hottest temperature occurs at the rightmost *x* value. B. The true gradient likely is some non-linear function of temperature $T(x)$. C. In A and B, we are assuming the gradient is invariant in the *y* direction, but in reality varying *y* describes a family of functions that are similar to $T(x)$. This family of functions, $S(x,y)$ describes the complete temperature plane.