Fundamental developments in larval zebrafish (*Danio rerio*) olfactory mediated behaviour

by

Angela Lee Shamchuk

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Physiology, Development and Cell Biology

Department of Biological Sciences
University of Alberta

Abstract

For many fish, their olfactory system allows for the critical detection of environmental chemical cues indicative of food, predators, kin and mates. Through odourant recognition, fish are able to react appropriately to their environment and elicit behaviours necessary for survival. The central objective of this thesis was to examine odourant-evoked behaviours of larval zebrafish (*Danio rerio*), who due to their age are not only ecologically vulnerable, but are undergoing rapid olfactory tissue development. Prior to these studies, few larval odourant-evoked behavioural responses had been investigated. Consequently, a portion of this thesis was dedicated to building the foundation for future studies. Two novel apparatuses were constructed: a flow-through system to observe changes in activity and an avoidance-attraction trough to test whether odourants affect area occupancy. Through validation of this equipment, behavioural responses to embryo extract (avoidance) and hypoxanthine-3-*N*-oxide (alarm) were characterized for the first time in 7 day-old larvae. To date, these are the earliest observed behavioural responses to these odourants in fish. Post-method validation, additional studies were conducted to identify 5, 6 and 7 day old larval responses to nucleobase compounds. While found to be behaviourally active, results indicated that nucleobase compound chemical structure, fish age and exposure naivety influenced occupancy behavioural responses. Overall, the work described in this thesis has expanded knowledge of nucleobase odourants, highlighted the importance of testing multiple behavioural metrics and established the much needed groundwork for future studies on larval olfactory mediated behaviours.

Preface

This thesis is original work by Angela L. Shamchuk and no part of this document has been previously published. Preliminary electrophysiology studies were conducted in collaboration with Brian Blunt. Synthesis and analysis of hypoxanthine-3-*N*-oxide was done in collaboration with Kirsten Tomlin of Dr. Jeff Stryker's laboratory. Glass chambers described in Chapter 2 and tanks described in Chapter 3 were custom built by Jason Dibbs. Plexiglas avoidance-attraction troughs were custom built by Jeffrey Johnston. The research herein described was approved by the University of Alberta Animal Care and Use Committee: BioSciences, AUP #052 'Olfactory Responses in Fishes'.

Dedication

"Always the beautiful answer who asks a more beautiful question."

— E.E. Cummings

This thesis is dedicated to my friends and family for their unwavering support of my research. I am forever grateful to have had so many coaches in my corner. Thank you especially to my parents David and Jayne for their love and encouragement. Their confidence in me has always been inspiring.

To Peter, I want to thank you for standing by me as we made yet another challenging climb. You've taught me what it means to persevere. The trail keeps going, but we can stop for a moment to enjoy the view.

Acknowledgments

This research would not have been possible were it not for the generous funding provided by both NSERC and DSM. Thank you to the members of my supervisory committee Drs.William Ted Allison and Declan Ali for lending their time and expertise towards my project. I am extremely grateful to my thesis supervisor Dr. Keith Tierney for his valued guidance and for ultimately affording me this opportunity for personal and academic growth. I must also acknowledge the numerous students that have worked with me on the material described in this document and on many other less fruitful ventures: Brian Blunt, Kirsten Tomlin, Patricia Leighton, Vlatka Vukojevic, Amy Wang, Anastasia Gasheva, Irtiza Oyon, Carlie Lewis and Elielma de Sousa. My sincerest gratitude is also extended to the Aquatics Facility Staff, notably Clarence Gerla, Aleah McCorry and Jesse Edgington for their unparalleled patience and support in helping me to establish our aquatics room. Finally, I greatly appreciate the equipment, favours and knowledge given to me by Jason Dibbs, Jeffrey Johnston, Troy Locke, Ben McDonald and Shelley Scott. This thesis is the culmination of the efforts and generosity of all of the phenomenal individuals listed here.

Table of contents

List of tables

List of figures

List of abbreviations

- Adenosine-5'-monophosphate: AMP
- Adenosine-5'-triphosphate: ATP
- Amino acid: AA
- Calcium-modulated photoactivatable ratiometric integrator: CaMPARI
- Centimetres: cm
- Correction factor: CF
- Cyclic AMP: cAMP
- Cytidine-5'-monophosphate: CMP
- Days post fertilization: dpf
- Embryo medium: EM
- Embryo extract: EE
- Food extract: FE
- G-protein coupled receptor: GPCR
- Guanosine-5'-monophosphate: GMP
- Heterogeneity chi-square: HCS
- Hours: hrs

- Hypoxanthine-3-*N*-Oxide: H3NO
- Inosine-5’-monophosphate: IMP
- Litres: L
- Millilitres: mL
- Millimetres: mm
- Minutes: min
- Molar: M
- Nucleotide: NT
- Olfactory sensory neuron: OSN
- Phospholipase C: PLC
- Region of interest: ROI
- Seconds: s
- Uridine-5’-monophosphate: UMP
- Versus: vs.

Chapter 1: Introduction

Significance of olfaction in fish

Olfaction, or the sense of smell, can be simply defined as the detection of chemical cues within the environment. While this description accurately identifies odourant detection as the critical step, interpretation and responses to sensory input should also be considered as essential components to a functional system. Is an odourant really an odourant without the induction of a physiological or behavioural response? If a compound holds no relevance to the detector, is it better classified as simply a neurostimulant?

Inclusion of the odourant response in this definition is essential because many of the evoked behaviours are linked to animal survival. Fish, in particular, rely on their olfactory abilities to sense and evade predators, locate food, navigate and breed [1]. Disrupted perception and responses to the environment can negatively impact survival and reproduction. Unfortunately, through human introduction of environmental pollutants, fish olfaction can be impaired or artificially stimulated [2, 3]. To better understand and prevent olfactory disruption, we must continue to investigate the complex composition and function of the fish olfactory system. This thesis endeavours to further characterize olfactory-evoked behaviours in a common genetic and toxicology model, the zebrafish (*Danio rerio*).

Fish olfactory system

In general, the fish olfactory system can be reduced to two key neural tissues: the rosettes and the olfactory bulb [4]. Each olfactory rosette is located within the nasal pit and is comprised of both sensory and non-sensory tissue. Water flows in and out of the anterior and posterior openings of the pit, which allows for solubilized odourants to encounter sensory tissue. The rosette is

described as an oval shaped structure with lamellae extending from a midline raphe [4, 5]. The olfactory epithelium is composed of receptor expressing olfactory sensory neurons (OSNs), support cells, and basal cells [5]. In zebrafish, it was found that there is no pseudo stratification of these cells types, meaning that they are not arranged in discernible layers within the epithelium [4]. Roles of support and basal cells remain largely unknown, although basal cells are thought to be involved in sensory neuron regeneration [6].

There are three classes of OSNs, ciliated, microvillous, and crypt, which differ in morphology as indicated by their names, and in their proposed odourant receptor population [4, 7]. There are five main odourant groups known to stimulate fish olfactory tissue: bile salts, nucleotides, amino acids, polyamines and pheromones [1, 8-15]. Ciliated neurons are less specific and have receptors for amino acids, bile salts and pheromones [16, 17]. Microvillous neurons however only have receptors for amino acids and potentially nucleotides [16, 17]. Crypt neurons represent a smaller population of OSNs and possess both microvilli and cilia structures [4, 5]. Crypt OSNs are less defined, however several studies have shown that they can be stimulated by amino acids and putative pheromones [18, 19]. The organization of OSN subtypes within the rosette displays no clear pattern, although in some species there are noted areas where lamellae have increased prevalence of singular OSN types [5]. Despite the random distribution within the epithelium, OSN axons that extend to the olfactory bulb as the bundled olfactory nerve exhibit mapped projection to target glomeruli [11, 17, 20, 21].

Teleost fish, including zebrafish, have four known families of G-protein coupled olfactory receptors (GPCRs): odourant, vomeronasal type I, vomeronasal type II, and trace amine associated [22, 23]. GPCRs have heterotrimeric G proteins meaning they contain a G protein complex of α, β and γ subunits. Exactly how odourant ligand binds to receptors is still

contentious. Amoore's 'key in a lock' theory describing the odourant perfectly fitting into the shape of the receptor is a persisting model, however as Brookes and colleagues (2012) point out, it does not account for receptors that are activated by multiple odourants [24, 25]. Brookes *et al*. instead suggest the 'swipe card' theory (heavily involving physics) in which the odourant structure has to match well enough to bind the receptor, but receptor activation depends upon odourant vibrational frequencies [25].

Regardless of the exact mechanism, after an odourant successfully interacts with an olfactory GPCR, there is a change in receptor protein conformation and subsequently GDP is exchanged for GTP on the G_{α} subunit [7]. This in turn causes dissociation of G_{α} from $G_{\beta\gamma}$. The G protein then goes on to activate secondary messenger transduction pathways via adenylate cyclase to produce cyclic AMP (cAMP) or by phospholipase C (PLC) to produce diacyl glycerol and inositol 1,4,5-triphosphate. Elevated secondary messengers stimulate cation influx, leading to OSN depolarization and possibly action potential generation. The signal is transduced along OSN axons to the olfactory bulb. At the olfactory bulb, OSNs synapse with mitral cells within distinct glomeruli. Odourant information is then interpreted and a physiological or behavioural response may be induced.

Through pharmacological manipulation, Hansen and colleagues have identified transduction cascades that are associated with distinct OSNs in catfish [16]. They found that ciliated neurons receptive to bile salts and amino acids operate via $G_{\alpha olf}$/cAMP and microvillous neurons receptive to amino acids operative via a $G_{\alpha q/11}$ / PLC pathway. However, there remains much that is not understood regarding fish olfactory receptor mechanisms. Nucleotide and polyamine odourants do not operate by either cAMP or PLC pathways, and they still have unidentified G proteins [8, 16]. There are also additional G proteins such as $G_{i\ 1b}$ expressed in larval zebrafish

olfactory tissue that currently have unknown odourant and OSN associations [26]. Furthermore, it is often overlooked that G proteins are not always conserved across fish species. For example, in catfish where $G_{\alpha o}$ is expressed in crypt neurons, it is expressed in goldfish microvillous neurons [16, 27]. Similarly, $G_{\alpha q/11}$ and $G_{\alpha q}$ are found in catfish microvillous neurons and crypt goldfish neurons [16, 27]. This generates doubt as to whether OSN classes should be generally ascribed to certain odourants as previously mentioned. A closer look at the complexity and diversity of fish olfactory tissue reinforces the importance of unique molecular and behavioural characterization per species.

Larval zebrafish as the experimental model

As previously mentioned, there can be significant variation between fish species in regards to olfactory tissue composition. Increased knowledge of the receptor repertoire and associated mechanisms within a single species will aide researchers in future comparisons across species. Zebrafish represent the best species to focus on due to their low cost and easy housing procedures, as well as the multitude of applicable genetic and molecular tools. While this thesis does not describe the molecular work required, by furthering the knowledge of behaviourally relevant compounds, questions regarding olfactory receptors and tissue become more directed. Specifically, characterization of odourant-evoked behaviour in larvae establishes a valuable endpoint for future loss-of-function studies such as olfactory receptor knockdown.

Larvae also represent the focal age in this study due to the rapid development of their olfactory tissue and relatively unstudied odourant-evoked responses (see Chapter 2 for further details). Zebrafish olfactory structures are electro-physiologically and behaviourally functional as early as 3 days post fertilization (dpf) [11, 28, 29]. Although, the system is functional during the larval stage, it is also highly dynamic during this time. The expression onset of different odourant

receptors has been observed to be asynchronous [30, 31]. Furthermore, after the additional onset, although overall receptor expression and olfactory bulb activity generally increase with progressive larval age, expression rates vary between odourant receptors [11, 30]. Suggested variable expression ratios between different receptor types during larval development may lead to changing capacity for odourant reception and interpretation. It is also necessary to consider that even though the olfactory tissue is capable of detecting a particular odourant, it remains to be seen whether that compound is behaviourally relevant. The intensity and type of behavioural response elicited may be influenced by mobility and/or a physiological need such as food, each of which increases with developmental stage [32, 33]. Taking these factors into consideration, we cannot assume that larvae in a vulnerable, naïve and dynamic life stage have the same chemosensory associations as an adult fish. Early development must be studied in order to determine how maturation affects behaviour.

Odourant-evoked behavioural endpoints

Although there are many different behaviours induced by odourant detection, this thesis will focus on two critical response categories: appetitive and alarm. Appetitive responses are those which include an increase in foraging and/or food ingestion [34, 35]. Foraging metrics vary across fish age and species, but are generally attributed to attempted food uptake or "pecking", changes in area use, increases in turn number and increases in activity [29, 34, 36, 37]. Being cues for sustenance, fish have positive associations with feeding stimulants and therefore many species are also attracted to odourant-concentrated areas [28, 34, 38]. An important caveat of feeding behaviour is that it is variable. Not all food odourants elicit both changes in activity and/or ingestion as well as chemical attraction [39].

Alternatively, alarm cues hold negative associations for fish because they are typically indicative of predator presence [40-42]. Fright responses have been characterized in several species as increases in erratic movement and periods of freezing, both of which can be methods of evasion [41-45] . As a sub-category to alarm-inducing compounds, there are odourants that serve as deterrents. Deterrents do not evoke the classically defined alarm response, but rather odourant avoidance [28, 34, 46, 47]. Both avoidance and fright behaviour are associated with the avoidance of danger, and would be expected to vary with the severity of the threat.

Since appetitive and alarm responses can both manifest as a change in activity or avoidance-attraction behaviours, it is important to investigate both endpoints when characterizing responses to novel odourants. Testing multiple compounds within an odourant class is also essential as there is precedent for different odourants of the same chemical category being able to evoke polar responses. Amino acids l-alanine and l-serine evoke appetitive and alarm responses respectively in fish [28, 34, 38, 48]. From studies demonstrating nucleotide-induced feeding and purine-evoked alarm, it is expected that nucleobase compounds as an odourant class also have both appetitive and alarm cues [35, 41]. Additional background information pertaining to olfactory-evoked behaviour and nucleobase compounds is in Chapters 2 and 3 respectively.

Thesis objectives

Principally, the objective of this thesis was to further the limited knowledge of larval zebrafish olfactory-evoked behaviour, specifically in regards to nucleobase compounds (see Introductions in Chapters 2 and 3 for background pertaining larval behavioural metrics and specific odourants). However to properly identify responses to nucleobase odourants, equipment needed to be developed for multiple behavioural assays. Research hypotheses are discussed further within each chapter.

Chapter 2

The primary aims of Chapter 2 were to: 1) develop and validate a larval zebrafish avoidance-attraction assay; 2) develop and validate a larval zebrafish flow-through odourant delivery system; and 3) identify behaviour-specific positive controls and thereby validate novel equipment. All objectives were met through successful equipment construction. Behaviour-inducing odourants were identified for both avoidance-attraction responses and activity analysis in 7 dpf larvae.

Chapter 3

Development of the avoidance-attraction trough enabled avoidance-attraction testing of nucleotides and other nucleobase compounds. Research questions included: 1) do nucleobase compounds evoke olfactory behaviours in zebrafish; 2) if so, do responses depend on nucleobase structure; 3) do behaviours vary with age; and 4) do repeated exposures diminish or strengthen responses? Objectives were met through larval nucleobase avoidance-attraction testing at 5, 6, and 7 dpf.

Chapter 2: Developing larval zebrafish methods for testing odourant-evoked behaviour

Introduction

The process of functional olfactory development in zebrafish presents an interesting and relatively unexplored area of research. Olfactory neural structure functionality and subsequent behavioural responses have been established as early as 3 dpf in zebrafish [11, 28]. The speed at which this sensory system develops reiterates its proposed importance to survival-mediated behaviour. Although most odourant classes are capable of activating the larval olfactory bulb, the behavioural significance of many compounds remains undefined (Table 2.1) [11, 49].

Both the type of behavioural response and the age of acquisition are important information in understanding innate vs. conditioned olfactory mediated activity. Despite operative neural stimulation, are some behavioural responses unique to specific life stages or experiences? Furthermore, how might responses differ between novel and familiar odourants?

In pursuing these research questions, it is key to be aware of the many changes in organismal activity that comprise a behavioural response. As it stands, in examining olfactory-mediated larval behaviour at least two central questions should be addressed: 1) does the subject exhibit aversion of or attraction to the odourant; and 2) does the odourant elicit a change in activity? As noted in Table 1, few studies have attempted to answer these questions for larvae. Therefore one of the primary research goals in this field should be testing odourants belonging to different classes to obtain a reliable catalogue of expected behavioural responses per developmental stage. Without this information, olfactory behaviour cannot be effectively used as an endpoint in either basic sensory system research or applied studies. In attempting to assemble a behavioural library of

Table 2.1. Odourants of interest for larval zebrafish behavioural investigation

Odourants	**Larval zebrafish behaviour (2-7 dpf)**	**Juvenile-adult zebrafish behaviour (post 7 dpf)**	**References**
Nucleotides	Unknown	Unknown, suspected feeding	[17, 35, 39]
Adenosine	Variable activity change, unknown association	Unknown, suspected feeding	[17, 50]
L-serine	unknown	Avoidance	[28]
L-cysteine	Avoidance	Avoidance	[28]
L-alanine	Increased activity	Attraction and feeding behaviour	[28, 36-38, 51]
Amino acid mixtures	Increased activity	Suspected feeding	[17, 29]
Chondroitin sulfate	Unknown	Alarm response	[42]
Bile salts	Concentration dependent attraction or avoidance	Attraction/Social	[28, 49]
H3NO	Unknown	Alarm response	[42, 45]
Skin extract	Unknown	Alarm response	[42, 44]
Polyamines	Unknown	Avoidance and stress	[46, 47]

Days post fertilization (dpf)

odourant-evoked responses, a second obstacle is apparent: there is a lack of simple, standardized and commercially available equipment and procedures that meet the specific experimental goals.

For avoidance-attraction testing of larval fish, there are several apparatuses that have been described. The primary issue has been that most designs have been created for larger fish and operate on a flow-through system. Typically fish acclimate in a flowing environment with two arms, and when flow in one arm is changed to include an odourant, time spent in the odourant zone can be observed [28, 47]. A flow avoidance-attraction apparatus has been created for larval fish, however it is not available for purchase and the small scale required presents design challenges that overwhelm the goal of having a simple and high throughput device [49]. A far simpler way to measure larval avoidance-attraction would be the use of a shuttle box, however these tests have only involved a built in light-dark preference or other external visual stimuli, and not exposure to a chemical stimulus [52, 53]. Odourant exposure without a flow-through system has been successfully performed by adding fluid to the centre of a larval group and measuring dispersal [28]. Although effective, the method was only applied to young, low activity embryos that could be moved and remain in the centre of the chamber. Moreover, fish were responsive to the simple addition of fluid to their environment which may overwhelm odourant reception. A more reliable and simple method would be a static trough where the fish could swim in and out of the areas where the odourant(s) had been added. Unfortunately, equipment with this function is neither commercially available nor previously described.

For exploring changes in swimming activity following odourant exposure, there are currently two methodologies available for larval zebrafish. One method involves the use of a static environment in which the odourant stimulus is added via pipette or injection [36, 50]. While olfactory responses may still be deduced, this method is not ideal because the addition of a fluid

to a static environment is in itself a stimulus. Larval zebrafish are able to detect changes in flow via their lateral line sensory system [54]. Such fluid addition can evoke a change in behaviour that masks any odourant-evoked activity [50]. Another method is to have continuous flow within the test environment so that odourant exposure is a single chemosensory stimulus that is unavoidable. This design was carried out by Lindsay and Vogt, in which a gravity fed flow-through petri dish was used with a group larval fish [29]. In this design, larvae can acclimate to a flow-through environment in which control water can be changed and odourant solutions introduced without disturbing the test atmosphere. While this group testing apparatus may be highly effective, several modifications would be necessary to effectively test individual larvae. Test chambers would need to be reduced in size and multiple chambers would need to be used simultaneously to increase experimental through-put. To strengthen consistency across trials and chambers, flow rates would need to be tightly controlled, which poses a challenge. Most importantly, the system should be enhanced so that within one experiment, fish can be exposed to multiple odourant classes consecutively, as well as a control water exposure. By allowing fish to be tested multiple times with different olfactory stimuli, a behavioural repertoire can be isolated for individual fish, which allows for better inclusion of individual variation.

To effectively test novel odourants, I determined it was necessary to develop novel equipment and a testing procedure. In doing so, I would also characterize several larval odourant-evoked responses for their potential use as positive controls in future studies. Subsequently, the initial objectives of this thesis were to:

1) **Develop and validate a larval zebrafish avoidance-attraction assay**
2) **Develop and validate a larval zebrafish flow-through odourant delivery system**
3) **Identify behaviour-specific positive controls through validation of novel equipment**

Methodology background and rationale

Testing age

For validation of novel larval equipment, 7 dpf zebrafish were used for three central reasons. First, it has been observed that zebrafish activity, quantified by resting time and area exploration increases with age [32]. Heightened baseline activity increases the subject's probability of stimulus encounters and allows for greater potential in deviations from normal activity. Additionally, post 7 dpf, feeding and consequently food odourant exposure, becomes necessary which removes naivety as a factor. Therefore, 7 dpf is the best test age for *novel* odourant exposure. Second, 7 dpf fish are well beyond the identified developmental threshold for olfactory-evoked behaviour and have functional swim bladders allowing for maximum movement potential (no spatial impediments) in both apparatuses [28, 29, 33]. Third, at 7 dpf embryonic yolk sacs, and therefore available sustenance, are depleted [33]. It is anticipated that, this stage will be the most responsive of the ages during the embryonic window to feeding cues due to physiological motivation.

Avoidance-attraction trough

The purpose of the avoidance-attraction trough is to accurately test for larval zebrafish odour-evoked changes in area use. The design allows for fish to move freely out of a middle chamber into odourant or water containing areas to the left or right of centre (see Materials and methods for more detail). The environment is controlled so that the entire area is visually identical (to fish) and no factor is present that will promote an area bias. Consequently validation of the apparatus depends on confirmation of two situational hypotheses: 1) under control conditions which lack olfactory stimuli, fish will distribute equally throughout the apparatus; and 2) the

presence of an aversive or attractive odourant will stimulate a shift in fish distribution so that occupancy per area is significantly different from an even dispersal. The experiments described demonstrate the validation of both hypotheses.

Flow-through system

The flow-through system was designed to test individual larval zebrafish responses to multiple odourants in a single stimulus environment. Since the flow-through experiment and the necessary track editing (see Materials and methods) are not rapid procedures, an underlying aim in analysis was to identify simple endpoints for which the results could be more or less automated using EthoVision XT tracking software (Noldus, NE). Subsequently, three simple behavioural endpoints were chosen to test the efficacy of the flow-through odourant delivery method: total distance travelled, time spent in the border zone and maximum velocity.

Total distance travelled was chosen as a metric for general level of activity and encompasses how much the fish has moved (mm) per unit of time. Total distance travelled was chosen instead of the commonly used measurement average velocity because of the bursting swim patterns exhibited by larval zebrafish. At 7 dpf, fish are not capable of continuous swimming and instead move in a stop-start or bursting manner [32, 55]. Due to the frequency of near zero values, the stop-starts alter observed average velocity and therefore the metric cannot accurately deduce high vs. low activity. Quantifying activity as number of bursts has been previously demonstrated as an effective metric; however the process requires further data manipulation than was desired for this experiment [50]. Based on previous larval and adult zebrafish studies, increases and decreases in activity, identified solely by an increase in distance travelled, could be indicative of either a fright or a feeding response [29, 34, 44, 56]. To further clarify whether an activity has a positive or negative association, other behavioural endpoints were included.

The amount of time spent in the border zone of the swimming chamber was chosen as an indicator of stress. Previous studies have shown that when anxious, larval zebrafish exhibit a preference for the edge, known as thigmotaxis [57-59]. Thigmotaxis behaviour was measured as time spent in the border zone established using an analysis template in the EthoVision software. I hypothesized that fear related odourants would cause an increase thigmotaxis.

The third endpoint, maximum velocity, was chosen in order to identify any dashing behaviour that may occur with odourant stimulus. Maximum velocity as an endpoint has been previously used to indicate startle response in zebrafish larvae [60]. If overall activity is depressed, this may also be demonstrated in lower maximum velocity. Minimum velocity was not chosen as a viable metric to identify decreases in activity because the aforementioned larval burst movement would result in a minimum velocity of zero in periods of both high and low activity.

Overall, these three behavioural metrics were chosen so that alarm responses could be clearly isolated when compared to other observed changes in activity.

Odourant selection

Embryo extract

Embryo extract (EE), which is homogenized and filtered zebrafish embryos, was chosen as a potential equivalent to the often utilized alarm compound(s) of 'skin extract'. Skin extract, also known as *Schreckstoff,* is released upon damage of epidermal club cells in certain fish species [61]. Skin extract evokes potent alarm responses characterized by erratic movement, shoal tightening, bottom dwelling, and slow swim/freezing episodes in many fish species, including adult zebrafish [42, 44, 59, 61]. The common hypothesis is that conspecific skin extract serves as an indication that a conspecific has been damaged by a predator [44]. Recently, Mathuru and

colleagues fractionated zebrafish skin extract and found that chondroitin sulfate (1μg/ml) was one of the behaviourally active components of skin extract [42]. Since then, chondroitin sulfate (1μg/ml) has been observed to effectively stimulate larval zebrafish olfactory tissue as early as 6-9 dpf, however behavioural responses of developing fish to any skin extract components are unknown [49].

Furthermore, although the embryo extract was prepared fresh, there are other odourants that are known for their association with decaying carcasses. For example, exposure to water from fish dead for 10 hours increases both behavioural and physiological fear related responses in zebrafish including freezing, bottom dwelling and whole body cortisol levels [46]. Cadaverine (a biological diamine associated with rotting tissue), in particular elicits an avoidance response in adult zebrafish and also can effectively stimulate larval olfactory tissue as observed through calcium imaging (10μM) [47, 49]. Oliveira and colleagues attribute the behavioural responses to carcasses to both the progressive decay of epidermal cells and subsequent *Schreckstoff* release, and to the diamine odourants [46]. It is currently unknown whether diamines are also components of skin extract.

The developmental stage for fully differentiated epidermal club cells has not yet been identified for zebrafish. However, if club cells are present, homogenization of whole embryos hastens the release of *Schreckstoff* as well as any biological diamines from the epidermal cells. Based on behaviours evoked in adult zebrafish, it was hypothesized that larvae would exhibit an aversive response to EE [42, 44, 46]. To ensure that sensory neuron detection thresholds had been reached, EE was tested at a high 1:10 dilution.

Food extract

Food extract (FE) was chosen as a feeding cue in order to stimulate an appetitive response. Calcium imaging demonstrated that FE (Tetramin; 1:10 dilution) was capable of stimulating zebrafish olfactory tissue as early as 3 dpf which is prior to development of active feeding [11, 62]. A different food brand was used in experiments described here (Larval AP100, Zeigler, USA), however the two products have comparable crude protein, fat, fiber, moisture and ash composition. A 1:10 FE dilution in embryo medium (EM) was used for the avoidance-attraction component of the study based on the calcium imaging findings. Although larval behavioural response to FE had not been specifically tested, it was predicted that larvae would demonstrate the same odourant attraction observed in adult zebrafish responses to FE and other feeding cues [38, 47]. Following trough experiments, flow through exposure concentration was reduced to 1:100 to lessen any negative response to the physiologically high concentration of FE. Within the flow-through apparatus, increased swimming activity, similar to that stimulated by amino acid feeding cues, was expected [29].

Amino acid mixture

An amino acid (AA) mixture was chosen as a potential positive control for the flow-through system because as already discussed, AAs are an established behaviour evoking odourant class for many fish species. In larval zebrafish, the olfactory bulb is activated as early as 2.5 dpf, by exposure to an amino acid mixture containing (1×10^{-4}M each) of alanine, methionine, histidine, lysine, tryptophan, phenylalanine and valine [11]. Importantly, responses to single AAs were also observed between 2.5-4 dpf. A simpler AA mix of alanine, cysteine and lysine ($1x10^{-4}$M each) also effectively stimulates the larval olfactory bulb (6-9 dpf) [49]. Physiologically, 7 dpf zebrafish should be able to detect AAs as the olfactory tissue is well developed [11, 30, 31]. Behaviourally, adult zebrafish classically exhibit an attractive or foraging response to L-alanine

[37, 38, 51]. Additionally, larvae also demonstrate an increase in swimming activity when exposed to a mixture of L-glutamine, L-methionine, L-alanine, L-cysteine, L-histidine, L-leucine, L-lysine, L-asparagine, glycine, L-serine, aspartic acid and glutamic acid ($1x10^{-4}$M each) [29]. Based on both the physiologically and behaviourally effective AAs identified, a mixture of L-phenylalanine, glycine, L-histidine and L-alanine ($4x10^{-5}$M total) was used for flow-through experiments. It is important to note that only positive, food associated AAs were desired for this mixture. L-serine and L-cysteine were not included in the mixture because zebrafish have previously responded to these odourants with avoidance [28]. It was hypothesized that the AA mixture would stimulate increased swimming behaviour, potentially identified by total distance travelled.

Hypoxanthine-3-N-oxide

In general, nitrogen oxides such as hypoxanthine-3-*N*-oxide (H3NO) have been demonstrated to be effective alarm compounds in several ostariophysan species including channel catfish (*Ictaluris punctatus*) and fathead minnows (*Pimephales promelas*) [43, 63]. Although predicted to be one component of skin extract, it is likely that H3NO contributes to a mixture of several fright inducing compounds in *Schreckstoff* [41, 42]. Nevertheless, it is extremely effective and in adult zebrafish specifically, H3NO induces fear-related behaviour characterized by erratic movements and jumping [42, 45]. The behavioural effects of H3NO on larval zebrafish have not been tested prior these experiments. However, based on fright behaviour evoked in adults, it was expected that H3NO exposure would induce an alarm response in larvae. A test concentration of $5x10^{-9}$M was chosen based on the most effective concentration in adults [45].

Nucleotide mixture

As previously discussed, nucleotides (NT) represent an identified class of odourants due to their ability to physiologically stimulate the olfactory tissue, but the type of behaviour elicited by nucleobase compounds in zebrafish remains undefined [7, 9, 10]. An NT mixture of adenosine triphosphate and inosine monophosphate ($1x10^{-4}$M each) is capable of stimulating olfactory bulb early in development (3-5 dpf) [11, 50]. Adenosine ($1x10^{-5}$M) exposure also induced variable activity changes in 5-7 dpf larvae [50]. Although generally hypothesized to be a feeding cue based on studies in invertebrates and other fish species, the purine ring structure in NTs bears resemblance to H3NO, and as such, they could also be fright-inducing substances (see Chapter 3 for more detail) [35, 39, 64, 65]. Conceivably, nucleotides could be released upon tissue damage, and therefore be unidentified components of skin extract. An NT mixture of uridine, adenosine, cytidine and guanine monophosphates ($4x10^{-5}$M total) was chosen as it included several potential behaviour-evoking structures including phosphates, sugars and a diverse array of purines. An NT mix was hypothesized to induce a change in activity associated with either feeding or fear behaviours.

Materials and methods

Zebrafish breeding and embryo rearing

Adult AB genotype zebrafish were housed in a specialized zebrafish aquatics rack (Aquaneering, USA) at the University of Alberta under institutional animal use protocol #052. The aquatics rack is a self-contained system that recirculates water though course filters, fluidized filter beads, carbon cartridges and UV sterilization. Fish were maintained at 28°C on a 14:10 light:dark cycle. Approximately 20L of fresh reverse osmosis water adjusted for conductivity and pH was added to the rack daily. Adult fish were fed a supplemented trout chow mixture (including blood worms, Omega Sea, China; spirulina flakes, Colbalt Aquatics, USA; and Tetramin flakes, Tetra,

Germany) twice daily. Zebrafish breeding pairs were separated by dividers until the morning breeding window to reduce temporal variation in development [66]. Embryos collected from breeding pairs were reared in embryo medium (EM) at 28.5°C under a 14:10 light:dark cycle until the desired testing age (5-7 dpf) [66]. The EM was refreshed daily and dead, or morphologically abnormal, embryos were removed. All behavioural trials, including pre-trial handling, were between the hours of 8am and 6pm, ensuring light exposure only within the rearing light cycle. In avoidance-attraction trials, odourant testing time was randomized to prevent temporal biases. Larvae have been previously found to exhibit greater baseline movement in the morning vs. the afternoon [67]. Following behavioural testing, larvae were transferred to the adult facility for further development or were sacrificed via rapid cooling [68].

Embryo medium

EM was prepared fresh weekly according to the recipe in [66] which consisted of : 20 ml Hank's Stock #1 (8.0g NaCl, 4.0g KCl, 100ml MilliQ H_2O), 2 ml Hank's Stock #2 (0.358 g Na_2HPO_4 anhydrous , 0.60 g KH_2PO_4, 100ml MilliQ H_2O), 20 ml Hank's Stock #4 (0.72 g $CaCl_2$, 50ml MilliQ H_2O), 1918ml MilliQ H2O, 20ml Hank's Stock #5 (1.23 g $MgSO_4x7H_2O$, 50ml MilliQ H_2O), 20ml fresh Hank's Stock #6 (0.7 g $NaHCO_3$, 20ml MilliQ H_2O). Solution was pH adjusted to 7.20 ± 0.05 using an Accumet AB150 pH probe.

Avoidance- attraction trough

Design and operation

The avoidance-attraction trough was a custom built Plexiglas apparatus for testing area use in response to odourants (Figure 2.1). The device measures 11 × 3.5 × 1.7cm (L × W × H) and contained removable inserts that divided the trough into three separate chambers. Trials were

recorded via an overhead camera (High resolution camera no. SX-920C-HR; Matco, Canada) connected to a PC running EthoVision XT version 8.5 recording software. The entire experimental setup was contained within a curtained chamber to limit disturbances from external stimuli [36]. During experiments, the apparatus was fixed to a raised platform with elastic bands. Once in place, the apparatus was levelled by an adjustable levelling stage fixed to the platform. Fifty ml of EM was added to the trough and the barriers were inserted. Experimental fish (n=9-11 per trial) in 7 ml of EM were transferred by pipette to the middle chamber of the apparatus. Water level across the three chambers was equalized and the test solutions (odourant or EM) were pipetted into adjacent chambers and mixed with the pipette tip.

Odourant solutions diffused equally throughout the designated side during the twenty minute acclimation (odourant side was randomized). Post-acclimation, barriers were carefully removed, causing little water disturbance, and fish movement was recorded for ten minutes. Apparatuses were rinsed for twenty minutes with distilled H_2O between trials to prevent odourant cross-contamination.

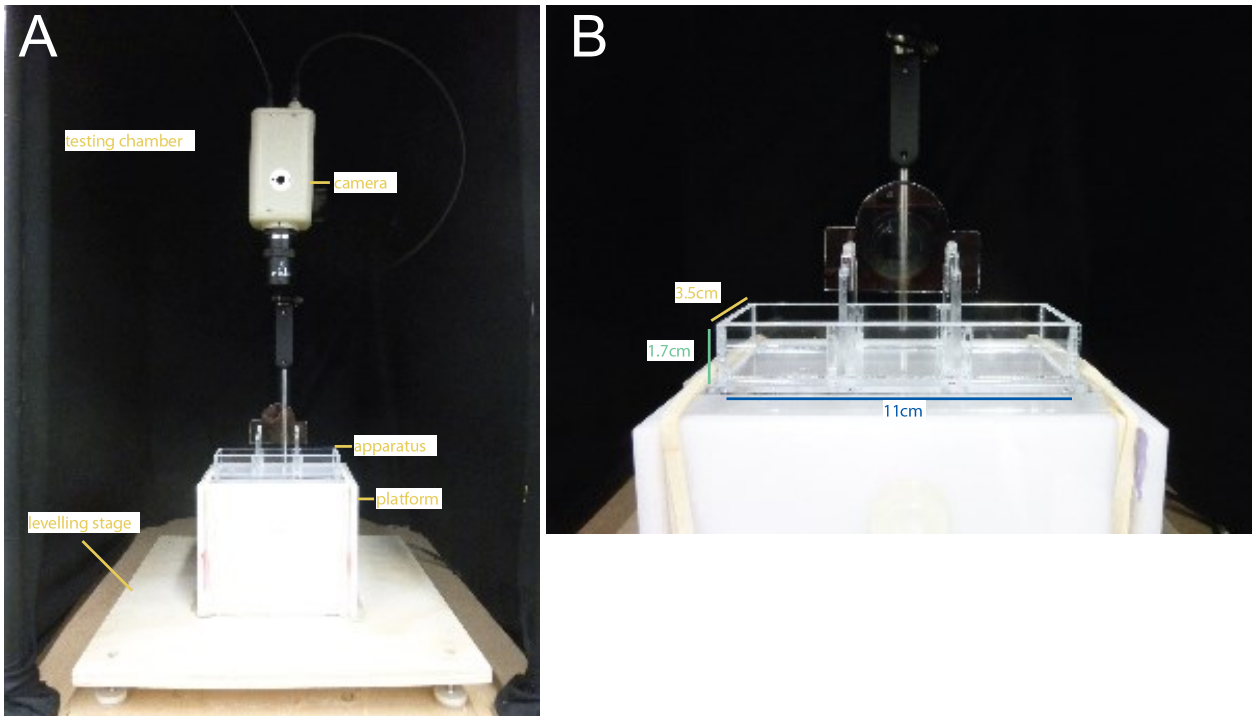


Figure 2.1. Avoidance-attraction apparatus experimental setup. A) Entire system within curtained enclosure. B) Close up and dimensions of apparatus.

Dye Trials

Dye trials were run prior to odourant trials to observe the prospective movement of odourants in the chamber. Red and yellow food colouring (Club House, Canada; final dilution $1x10^{-3}$) were used instead of odourant solutions in simulated trials with n=10 7 dpf larvae per trial (n=12 trials, 6 per apparatus; Figure 2.2A). Regions of interest (ROIs) were made in ImageJ (National Institutes of Health, USA) by identifying dye infused areas at each time point to be scored (10, 20, 30, 45, 60, 90, 120, 180, 240, 300, 420 and 600 s). Final visual overlays of the dye area were created by combining the ROIs from each trial. For example, for each apparatus, the left ROI at 10 s was a shape made from combining six images. The final ROIs were used as overlays to facilitate manual scoring of fish position (Figure 2.2B).

7 dpf apparatus validation

To validate apparatus efficacy in avoidance-attraction testing, four sets of experiments were conducted, in which fish were exposed to: EM (control), EE vs. EM, FE vs. EM and EE vs. FE (n = 8 trials per condition; n = 9-11 fish per trial).

Odourant preparation

Embryo extract: Ten 7 dpf embryos were anesthetized and sacrificed via rapid cooling and homogenized for 1 minute (min) in 1ml of EM. Homogenate was filtered and the filtrate volume was adjusted to 1ml. EE was made fresh prior to trials. Final apparatus dilution was 1:10.

Food extract [11]: 200mg larval food powder (AP100, <100microns; Zeigler) was incubated in 50mL of EM at room temperature 1 hr and the mixture was filtered and stored at 4°C. Prior to experimental testing, extract was brought to room temperature and tested at a 1:10 dilution. FE was made fresh weekly.

Manual scoring and statistical analysis

Fish position was scored using screenshots of each trial at 10, 20, 30, 45, 60, 120, 180, 240, 300, 420 and 600s post barrier removal. Screenshots were adjusted in Adobe Photoshop CS4 (Adobe Systems, USA) to standardize image size and orientation prior to use in ImageJ. ROIs specific to each apparatus, side (left or right), and time point were overlaid on screenshot images. The number of fish per left, middle and right zone, as well as odourant vs. non-odourant zones, was recorded. Fish spanning ROI boundaries were scored based on head position. Inter-rater reliability between the three manual scorers was validated via a two way mixed model of absolute agreement intraclass correlation coefficient test (ICC) for three trials in SPSS (IBM,

USA) Left side scoring ICC(3, 3) =0.997 and right side scoring ICC(3,3)=0.987 confirmed reliable scoring across individuals.

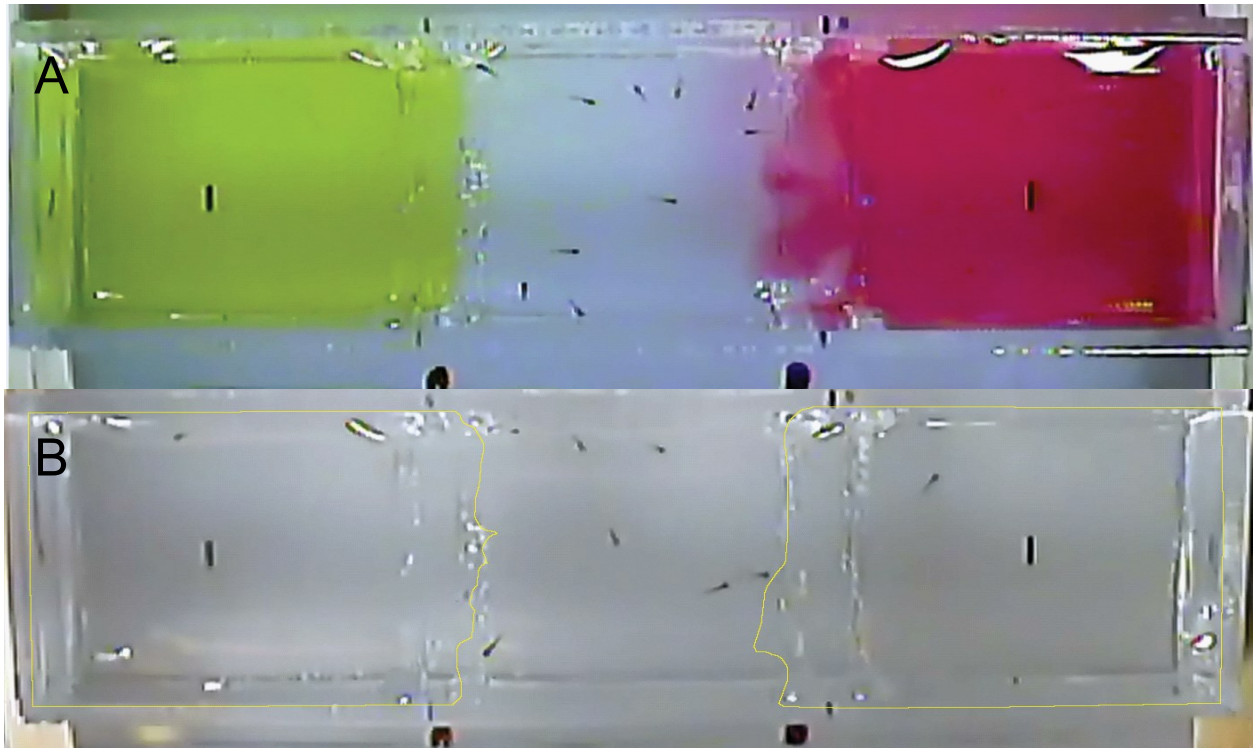


Figure 2.2. Sample avoidance-attraction dye trial and region of interest (ROI) overlay. A) Red and yellow food colouring dye were used to simulate odourant solution diffusion within the apparatus under trial conditions (7 dpf; n=10). B) ROIs denoted by the yellow line identify the combined area where dye is present across each trial (n=6 per apparatus). Combined ROIs per time point of interest produce a final overlay used to determine fish presence in odourant (dye) vs. non-odourant (no dye) areas for manual scoring.

Fish distribution across the apparatus was determined using heterogeneity chi-square (HCS) analysis [69]. The trials determined to have acceptable homogeneity were pooled and values were corrected for continuity (Yates correction). Time points analyzed in odourant trials were limited to points that followed a 1/3:1/3:1/3 distribution in EM trials. Odourant vs. EM trials were analyzed for a corresponding 1/3:2/3 odourant vs non-odourant zone distribution via HCS analysis. Odourant vs. odourant trials were analyzed using 1/3:1/3:1/3 HCS analysis. Data is presented as mean ± SEM.

Flow-through apparatus

Design

A flow-through apparatus was custom built that facilitated switching between delivery fluids without disturbing flow to the chambers (Figure 2.3). Odourant solutions for delivery were held in 60ml plastic syringes in a 28.5°C water bath (i.e. at the same temperature as the embryos). Water bath temperature was maintained by circulating water from a Lauda (Alpha RA 8; USA). Due to the volume of EM used, a separate stock of EM was stored in the central Lauda bath and pumped via a three roller peristaltic pump (Cole Parmer; Canada) to the odourant delivery syringe. A Masterflex speed controller (Cole Parmer; Canada) ensured maintenance of a constant flow rate and subsequent head pressure within the EM syringe. All syringes had overflow ports to maintain a constant volume. Since odourant storage components were plastic, each syringe was designated for a specific odourant to reduce cross-contamination. Odourant syringe tips extended outside of the water bath and were connected to 3-channel stopcocks which controlled flow and enabled line priming and flushing. 18 ½ gauge needles (PrecisionGlide; BD, Canada) connected the stopcocks to odourant lines (polyethylene tubing 190; BD, Canada). Fluid lines flowed to individual drip chambers to eliminate air bubbles within the system. From the drip

chambers, odourant lines flowed through a c-flex tubing section (1/32 ID; Warner, USA) threaded through individual solenoid valves. Valves were controlled by manually operated switches on a six channel valve controller (VC-6; Warner, USA). The six lines from solenoid valves fed into an 8-into-1 micromanifold (AutoMate; USA). The chosen solution flowed via gravity from the manifold into a double funnel system. The solution flowed primarily into the small interior funnel which fed into a 1-into-4 micromanifold (AutoMate; USA). To maintain constant head pressure, fluid was allowed to overflow into the exterior funnel and was discarded as waste. From the micromanifold, the apparatus had four odourant lines that each connected to sections of Masterflex silicone tubing (Cole Parmer; Canada). The silicone tubing ran through a speed controlled peristaltic pump (Masterflex; Cole Parmer; Canada) and was then reconnected to polyethylene odourant lines. All tubing connections were threaded by pipette tip inserts and sealed by parafilm wrappings. Odourant lines fed into a second drip chamber to eliminate any newly formed air bubbles before connecting to the custom stoppered glass chambers (3ml volume; 19mm in diameter). Each chamber held a single fish, therefore n=4 maximum per trial. Odourant solutions flowed into the lower inflow arm and out the upper outflow arm of the chambers (Figure 2.3, inset). Chamber arms were narrowed at the openings and had silver wire (0.250mm; World Precision, USA) inserted to prevent larvae from leaving the chamber. Outflow tubing led to an exterior waste bucket. Chambers were inserted in an elevated foam section where they were secured with an elastic band. White paper was placed above the chambers to reduce the intensity of the overhead light (Globe full spectrum bulb, 750Lumens). Fish movement was recorded from below with a tripod fixed JVC HD Everio video camera (JVC; USA). To prevent experimenter interference, the camera was controlled wirelessly by the

Everio sync.2 app (free downloadable app) on a portable device. During testing, the chambers were surrounded by a black curtain to prevent disruption from external environment.

Operation

Prior to experiments, individual chamber flow rate was calibrated to 2.3ml/min (rate determined by preliminary larval motion observation within the chambers) and EM was set as the background flow-through solution. A single 7 dpf larval zebrafish was transferred to each chamber and the chambers were manipulated to remove air bubbles. Chambers were fixed in the elevated foam block and orientated for optimized camera viewing. Chambers were then enclosed within the curtain and the 20 minutes post transfer acclimation began (Figure 2.4). During this time odourant solutions (AA, H3NO, NT, EM, and FE) were prepped and added to their designated syringes to reach testing temperature. Post-acclimation, five minutes of baseline activity was recorded followed by one and a half minutes of odourant #1 delivery via solenoid valve control. After the one and a half minutes, background EM flow was returned and activity was recorded for another eight and a half minutes (total of ten min exposure activity). Based on dye trials, odourant washout was complete eight minutes into the trial (six and a half minutes post dye delivery). From washout, fish were given ten minutes of post-stimulus re-acclimation. Once re-acclimated, five minutes of baseline activity was recorded followed by one and a half minutes of odourant #2 delivery. The washout-delivery process was repeated for five odourants. In this experiment odourants #1-5 were AA, H3NO, NT, EM and FE respectively (n=10).

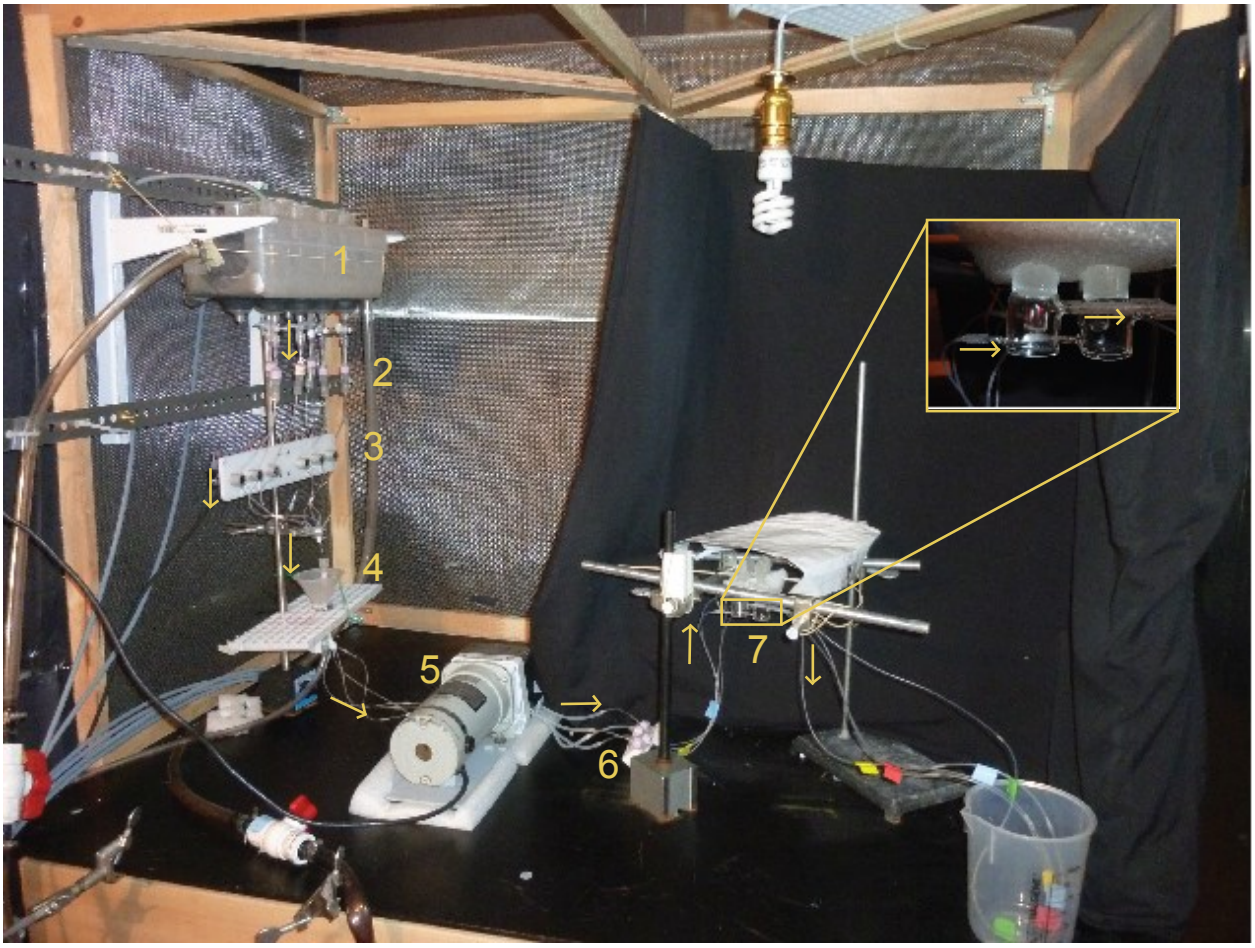


Figure 2.3. Flow-through apparatus schematic. 1) Odourant solutions are held in a 28.5°C water bath. 2) Syringes containing odourant solutions are connected to stopcocks. Post stopcocks, odourant lines flow to drip chambers. 3) Odourant lines flow through individual solenoid valves. 4) From solenoid valves, lines feed into an eight channel manifold. Odourant solution flows from the manifold into a double funnel system. The interior funnel solution connects to a four channel manifold. 5) Flow from the four odourant lines is controlled via a peristaltic pump calibrated to produce a 2.3mL/min flow rate per line. 6) Odourant lines feed into a second drip chamber. 7) Odourant lines connect to the elevated custom glass chambers. White paper is placed above the chambers to reduce the intensity of the overhead light. Inset: Odourant solutions flow into the lower arm and out the upper arm of the chambers. Chamber arms have silver wire inserted to prevent larvae from leaving the chamber.

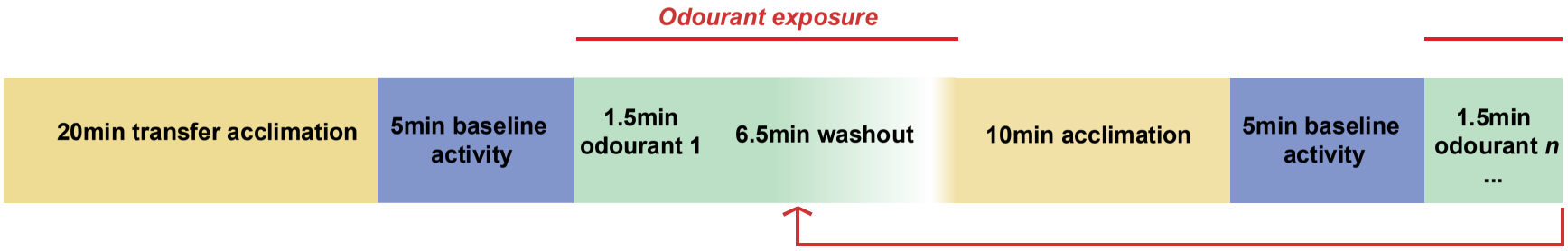


Figure 2.4. Flow-through apparatus trial procedure. 7 dpf zebrafish underwent this trial procedure for odourants 1-5: amino acid mixture (AA), Hypoxanthine-3-N-oxide (H3NO), nucleotide mixture (NT), embryo medium (EM) and food extract (FE). During acclimation periods background flow was embryo medium (EM).

Flow calibration

Red food colouring dye ($1x10^{-2}$ dilution; Loretta, Canada) simulated odourant solution delivery as previously described to determine estimates for final odourant exposure concentration and washout time. Samples were taken from pre-trial chambers (distilled water only) and during the progression from dye delivery to washout (0.5-16 min; every 30s until 5 minutes and every minute until 16 minutes; Figure 2.5). The absorbances for trial samples (n=3 trials) and standard curve samples (2 sample replicates per plate, n=3 plates) were measured at 504nm using a 96 well plate reader (optimum wavelength previously determined; data not shown). Maximum chamber dye concentration during trials was calculated from the standard curve (Figure 2.6). The dilution factor obtained was used to correct odourant stock delivery concentrations. Kruskal-Wallis one way ANOVA on ranks determined that dye washout was effective at 8 min (H_{21}=254.506, p<0.001, Tukey: 0.5 min vs. 8 min p>0.05; Figure 2.7).

Odourant preparation

Nucleotide mixture (NT): UMP (Uridine 5′-monophosphate disodium salt; U6375, Sigma-Aldrich; ON, Canada), AMP (Adenosine 5′-monophosphate monohydrate; A2252, Sigma-Aldrich; ON, Canada) CMP (Cytidine 5′-monophosphate disodium salt; C1006, Sigma-Aldrich; ON, Canada) and GMP (Guanosine 5′-monophosphate disodium salt; G8377, Sigma-Aldrich; ON, Canada) were combined in equal parts (1:1 ratio) for a final stock concentration of $4x10^{-3}$M in EM. Stock solution was pH adjusted to 7.20 ± 0.05, aliquoted and stored at -20°C. Aliquots were thawed and experimental solutions were made in EM immediately prior to testing for a final exposure concentration of $4x10^{-5}$M. Freezing of odourant stock solutions did not reduce their efficacy [45]. Test concentration was determined from preliminary electro-olfactogram studies (Blunt, unpublished; data not shown).

Amino acid mixture (AA): L-phenylalanine, Glycine, L-histidine and L-alanine (Sigma-Aldrich) were combined in equal parts for a final stock concentration of $4x10^{-2}$M in EM. Stock solution was pH adjusted to 7.20 ± 0.05, aliquoted and stored at -20°C. Aliquots were thawed and experimental solutions were made in EM immediately prior to testing for a final exposure concentration of $4x10^{-5}$M.

Food extract (FE): Extract was prepared as previously described in avoidance-attraction odourant preparation, however final flow-through testing dilution was 1:100.

Hypoxanthine-3-*N*-oxide salt (H3NO): Hypxanthine-3-*N*-oxide (H3NO) was synthesized from 6-methoxypurine as previously described [45, 70]. 6-methoxy-purine was first converted to 6-methoxypurine-3-*N*-oxide and then to H3NO. The chemical identity of the H3NO product was verified via NMR, elemental analysis and mass spectrometry. Mass spec identified the cation product molar mass to be 152.0334g/mol, which is consistent with a commercial grade product and the chemical identity. Synthesized H3NO was a salt with a Cl^- anion, therefore total molar mass is 188.57g/mol. $5x10^{-7}$M H3NO in EM stock solution was pH adjusted to 7.20 ± 0.05, aliquoted and stored at -20°C. It was essential that H3NO solution be prepared in pre-buffered EM (pH 7.20 ± 0.05) and then subsequently pH adjusted because the H3NO chemical structure is most stable at neutral or slightly alkaline pH [45, 71]. Acidic exposure alters the N-O functional group and renders the compound behaviourally inactive. Experimental solutions were made from thawed aliquots in EM immediately prior to testing for a final exposure concentration of $5x\ 10^{-9}$M. The test concentration was previously determined to be the most effective concentration tested in adult zebrafish behavioural trials [45].

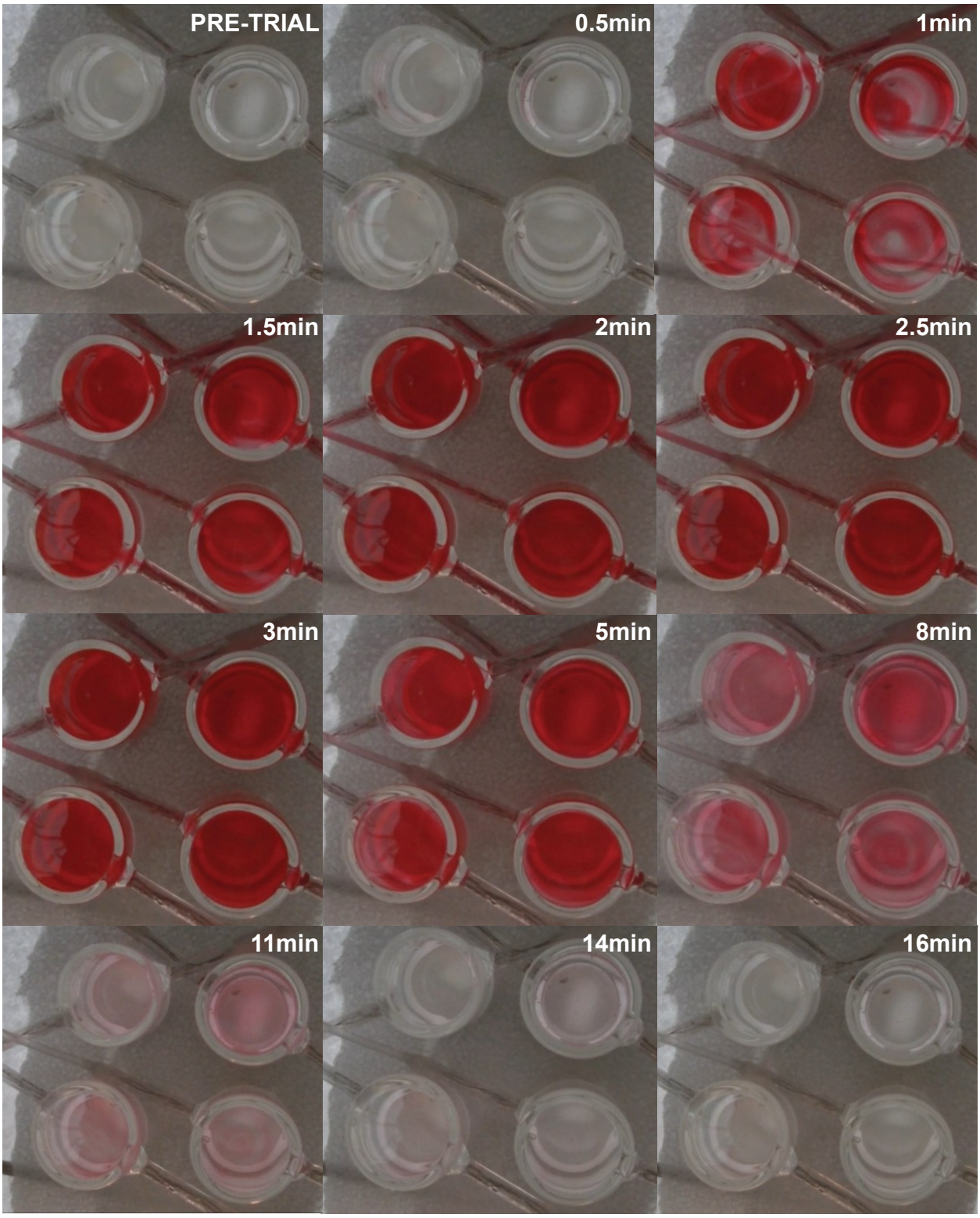


Figure 2.5. Sample time course images from a dye trial. Red food colouring dye ($1x10^{-2}$) was used to simulate odourant solution delivery. Images above show pre-trial chambers (water only) and the progression from dye delivery to washout (0.5-16 min).

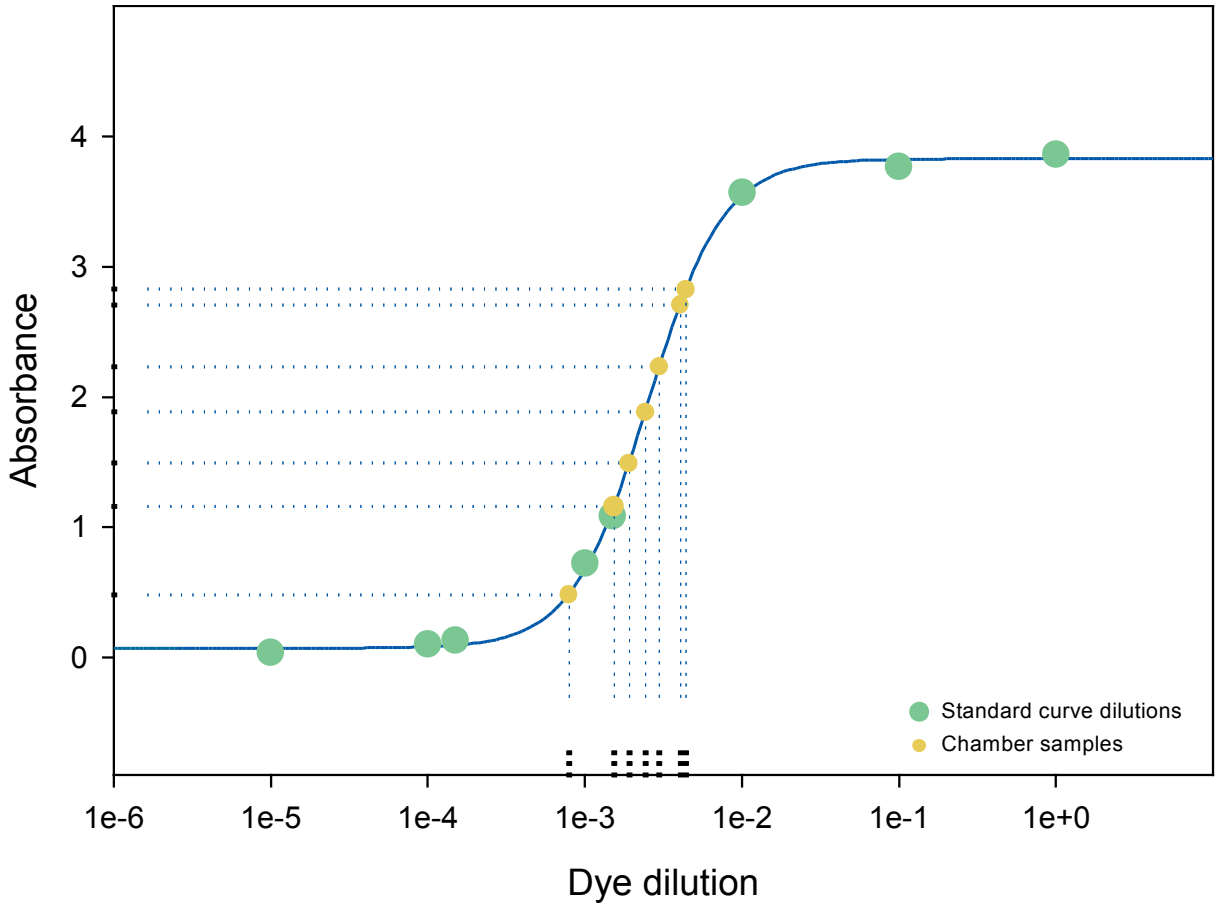


Figure 2.6. Dye calibration standard curve. Absorbances of known dilutions of dye were measured using a plate reader at 504nm to produce a standard curve (2 sample replicates per plate, n=3 plates). The standard curve enabled dilution calculations for outflow samples obtained during dye trials.

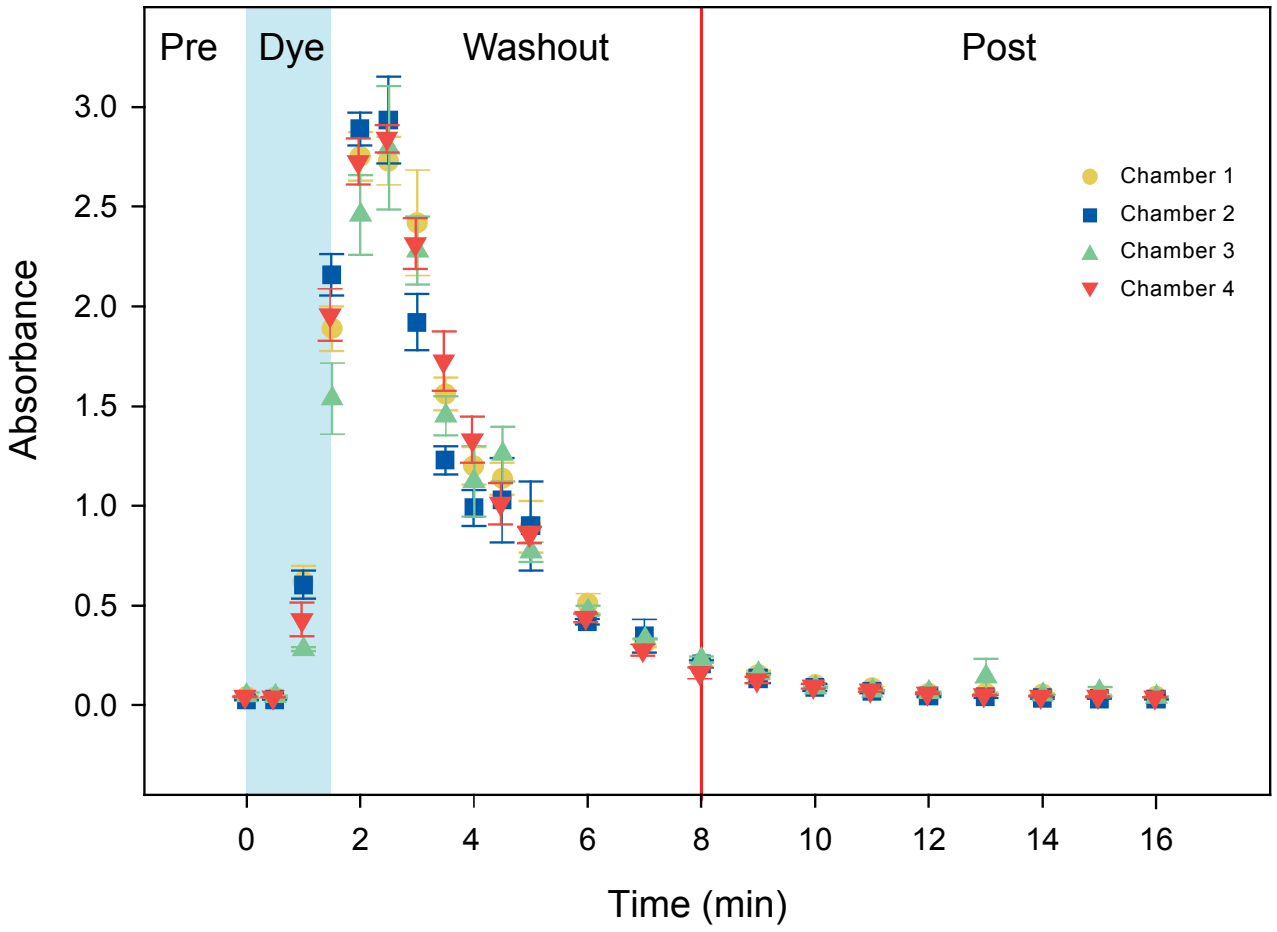


Figure 2.7. Absorbances of chamber outflow samples during dye trials. Plots represent mean absorbance values ± SEM from each chamber at select time intervals (n=3 trials). Absorbances were measured at 504nm using a plate reader. Blue area represents dye delivery period (1.5min). Red line denotes end of washout period as the sample was not statistically different from pre-dye addition.

Behaviour and statistical analysis

Fish movement in flow-through trial videos were tracked using EthoVision XT version 10.1. Arenas were constructed to include both border and Centre zones of the chamber (Figure 2.8) Fish were tracked under dynamic subtraction at ~30 samples per second. The acquired track of movement was edited to correct for missing and off target samples. Behavioural metrics examined include total distance travelled (mm), time spent in border zone (s) and maximum velocity (mm/s). Data is presented as mean ± SEM.

Unless otherwise noted, statistical analysis was performed and graphs were created using SigmaPlot 11.0 (Systat; CA, USA). Statistical significance was generally set at $p<0.05$, however there are certain comparisons where *p*-values near this threshold were reported. All reported *p* values have been adjusted from SigmaPlot unadjusted *p* value output. Non-parametric data was rank transformed prior to parametric testing as suggested by Conover [72]. Average distance travelled (mm) during baseline exposures across the trial was analyzed using a Friedman repeated measures ANOVA on ranks. Changes in activity during odourant exposure were analyzed by calculating each individual fish's percent change from baseline activity per minute of exposure; positive and negative values indicate increases and decreases in distance travelled respectively. Percent changes during AA, H3NO, NT and FE exposure were each compared to EM percent changes using a two way repeated measures ANOVA and Holm-Sidak post hoc on rank transformed data. Total time spent in border zone during five minutes of baseline activity was compared across the 5 baselines intervals using a Friedman repeated measures ANOVA on Ranks. Time spent in border zone during odourant exposure was compared to border use during EM exposure using a two-way repeated measures ANOVA and Holm-Sidak post hoc on rank transformed data. Notably, average time spent immediately prior to baseline activity was also

used in this comparison as 1 min. Maximum velocity during baseline activity periods across trials were analyzed using a one way repeated measures ANOVA. The trial was then divided into pre exposure (five minutes of baseline activity), exposure (8 min of odourant presence) and post exposure (two minutes post washout activity). Maximum velocities per interval were compared using a two way repeated measures ANOVA and Holm-Sidak post hoc.

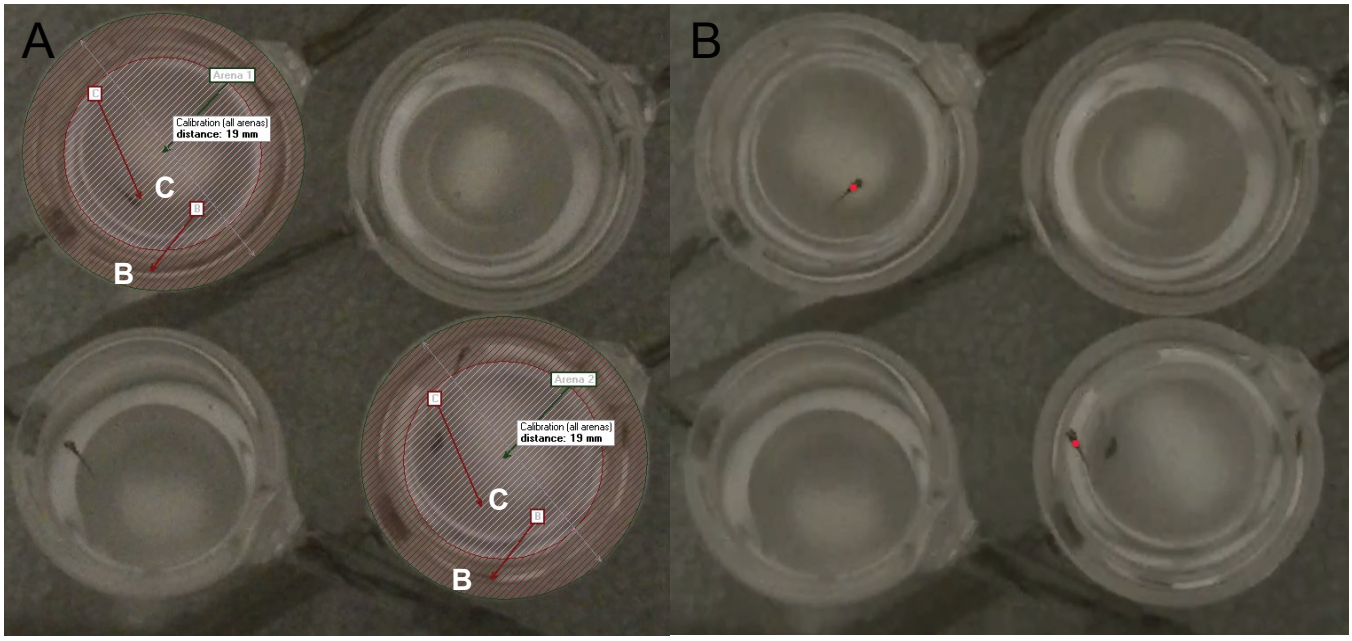


Figure 2.8. Sample image of EthoVision XT arena set up and tracking. A) Arena settings. White B and C letters indicate border and centre zones respectively. Chamber image is calibrated to real life diameter of 19mm. B) Screenshot of movement tracking in progress. Red marker identifies centre point of the fish.

Results

Avoidance-attraction trough validation

Fish area occupancy under control conditions

During control trials where EM was used as an odourant, initially larvae did not exhibit the expected 1/3:1/3:1/3 even distribution across the trough (Figure 2.9) Fish instead mainly occupied the middle chamber at time points 10, 20, 30, 45, 60, 90 and 120 s, having on average 76.3 ± 5.7, 71.3 ± 4.8, 65.0 ± 5.7, 58.8 ± 6.9, 53.8 ± 7.5, 52.5 ± 7.5 and 46.3 ± 5.3% occupancy respectively vs. the expected 33.3% (n=8 trials, 10 fish per trial; left vs. middle vs. right heterogeneous chi square test : HCS $\chi^2_{0.05,\,14}$ = 10.81, 9.74, 10.04, 16.77, 16.61, 16.53, 11.80, pooled corrected $\chi^2_{0.05,\,2}$ =66.93, 52.68, 28.55, 25.98, 18.03, 13.98, 10.43).

By 180, 240, 300, 420 and 600s, larvae were distributed evenly across the three chambers of the trough and middle chamber mean occupancy was to 41.2 ± 6.1, 33.8 ± 6.8, 32.5 ± 6.5, 28.8 ± 4.8 and 23.8 ± 6.0%, respectively (left vs. middle vs. right heterogeneous chi square test: HCS $\chi^2_{0.05,\,14}$ = 13.44, 15.09, 17.19, 12.54, 12.39; pooled corrected $\chi^2_{0.05,\,2}$ =3.23, 1.20, 0.93, 1.30, 3.68).

Mean occupancy on the left side of the trough for 180, 240, 300, 420 and 600s was 35.0 ± 5.7, 38.8 ± 4.8, 38.8 ± 5.8, 40.0 ± 5.3 and 42.5 ± 5.3%. Mean right side occupancy for the same time periods was 23.8 ± 3.2, 27.5 ± 4.5, 28.8 ± 5.2, 31.3 ± 4.8 and 33.8 ± 3.2%. Although there appears to be slight bias towards the left side of the apparatus near the end of the trial, it was not a great enough difference to violate the expected distribution.

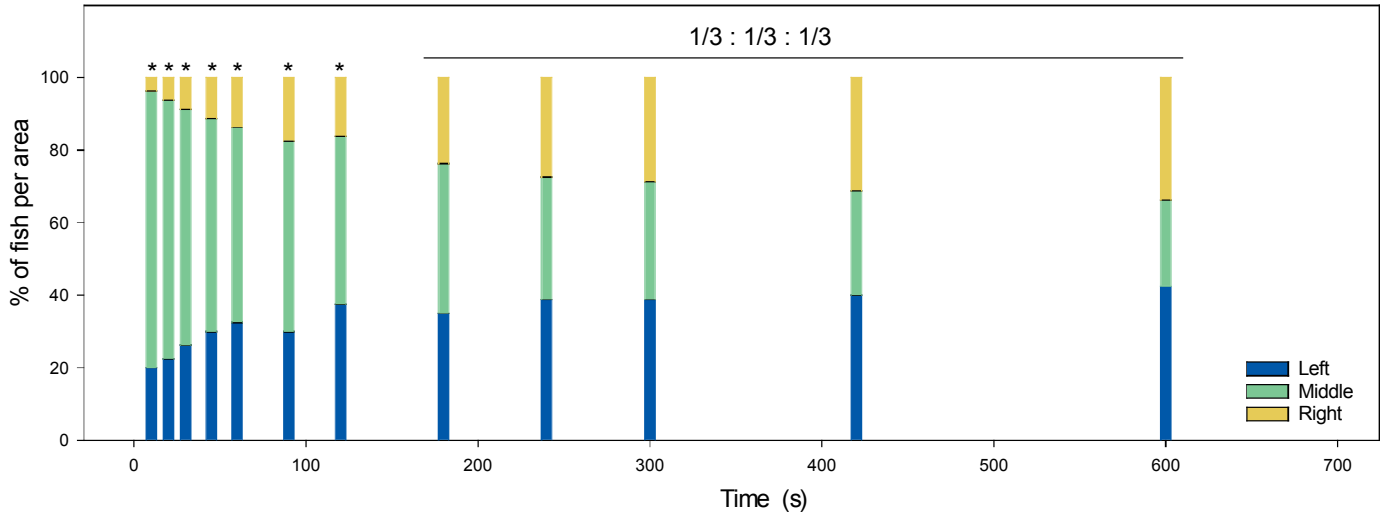


Figure 2.9. Percent occupancy per region for 7 dpf larvae under control EM conditions. EM was used on each side of the apparatus. Following divider removal the number of fish present in each ROI was manually scored at selected time points. Bars illustrate average percent of fish per area (n=8 trials, n=10 fish per trial). Asterisks denote time points that are significantly different from an equal (1/3:1/3:1/3) distribution.

Larval avoidance-attraction behaviour in response to odourants

Only time points where even distribution was observed under control conditions were used in analysis of odourant trials, therefore all reported results refer to 180, 240, 300, 420 and 600s in that order. The EE (1:10) exposed fish displayed a strong avoidance to the odourant side with only 9.9 ± 3.3, 9.9 ± 3.3, 9.8 ± 2.6, 13.6 ± 5.0 and 17.4 ± 5.3% mean occupancy compared to the expected 33.3% (Figure 2.10A). Odourant vs. non-odourant area heterogeneous chi square testing confirmed a significantly skewed distribution at each time point (n=8 trials, 10-11 fish per trial; HCS $\chi^2_{0.05,\,7}$ = 2.67, 2.67, 1.64, 6.26, 7.05; pooled corrected $\chi^2_{0.05,\,1}$ = 19.01, 19.01, 19.01, 13.35, 8.68). Interestingly, the non-odourant area did not exhibit equal fish distribution between the middle and opposite odourant side, with the opposite side having a mean percent occupancy ≥50% at each time point (49.6 ± 5.2, 55.7 ± 5.8, 53.4 ± 5.9, 64.4 ± 7.0 and 66.7 ± 5.9%).

The FE (1:10) exposed fish however, did not show increased occupancy in any chamber and met the expected 1/3:2/3 distribution (Figure 2.10B; n=8 trials, 9-10 fish per trial; odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,7}$ = 4.28, 5.13, 4.83, 3.92, 5.52; pooled corrected $\chi^2_{0.05,\,1}$ = 0.01, 0.01, 0.70, 0.01, 0.01). The FE side had 32.9 ± 4.1, 34.2 ± 4.6, 27.8 ± 4.5, 31.7 ± 4.0 and 33.1 ± 4.7% mean percent preference. The odourant opposite side had a mean preference of 33.9 ± 5.2, 31.5 ± 3.9, 37.8 ± 5.1, 42.8 ± 5.4 and 44.2 ± 3.0%. The middle chamber had a corresponding mean percent preference of 33.2 ± 5.0, 34.3 ± 4.4, 34.4 ± 5.3, 25.6 ± 4.5 and 22.8 ± 2.5%. Notably, time points 420 and 600s showed a greater percentage of fish in the opposite side vs. the middle.

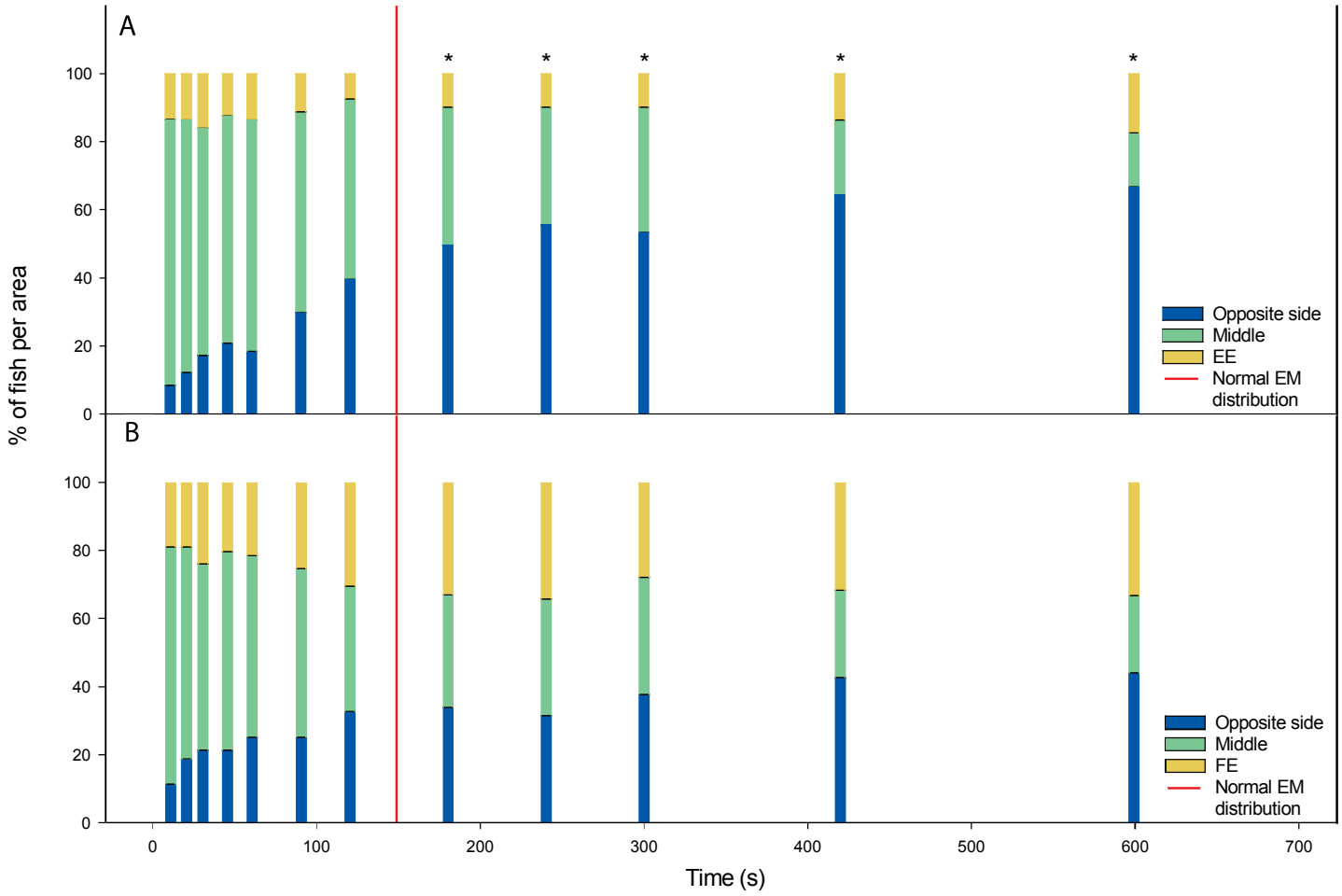


Figure 2.10. Percent occupancy per region for 7 dpf larvae in odourant vs. EM trials. A) Embryo extract (EE; 1:10) or B) food extract (FE; 1:10) was present on one side of the apparatus while EM was on the other (odourant side was randomized across trials). Bars illustrate average percent of fish per area (n=8 trials per odourant, n=9-11 fish per trial). Red line denotes point of equal distribution of fish in control trials. Asterisks indicate a significant difference from an equal (1/3:2/3) distribution observed in control trials; heterogeneous chi square test.

Fish exposed to EE on one side and FE and the other also showed a shifted distribution from the expected 33.3% per chamber (Figure 2.11; n= 8 trials, 10 fish per trial; odourant 1 vs. middle vs. odourant 2 heterogeneous chi square test: HCS $\chi^2_{0.05, 14}$ = 9.85, 14.20, 19.61, 14.95, 5.35; pooled corrected $\chi^2_{0.05, 2}$ = 11.70, 10.43, 12.78, 11.43, 21.78). EE had a less than expected mean percent occupancy across the trial 15.0 ± 5.0, 16.3 ± 4.6, 17.5 ± 6.2, 20.0 ± 5.0 and 20.0 ± 3.3%. These values were however greater than those observed when EE was tested alone. The FE side also had greater occupancy than when tested alone with a mean percent occupancy of 46.3 ± 3.8, 46.3 ± 5.7, 51.3 ± 5.2, 51.3 ± 4.8, 58.8 ± 4.0% across the trial.

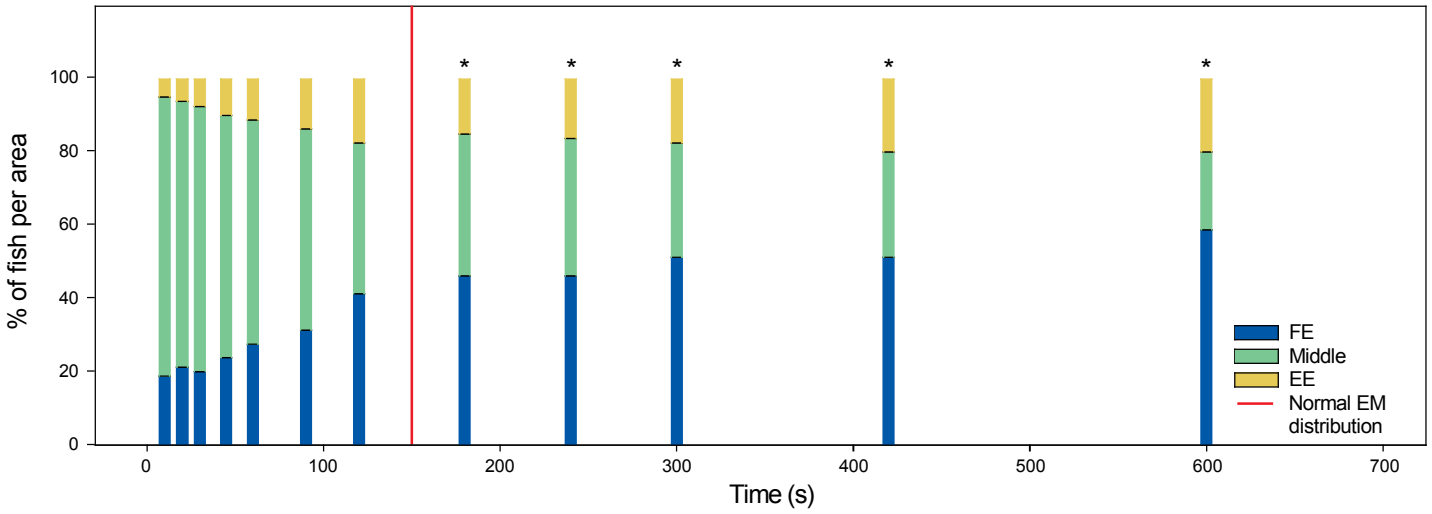


Figure 2.11. Percent occupancy per region for 7 dpf larvae in food extract (FE) vs. embryo extract (EE) trials. EE (1:10) and FE (1:10) was present on opposite sides of the apparatus (odourant side was randomized across trials). Bars illustrate average percent of fish per area (n=8 trials per odourant, n=10 fish per trial). Red line denotes point of equal distribution of fish in control trials. Asterisks indicate a significant difference from an equal (1/3:1/3:1/3) distribution observed in control trials.

Flow-through data

Distance travelled

There was no difference in the average distance travelled (mm) during the five minutes of baseline activity prior to each odourant exposure (Figure 2.12; n=10; Friedman repeated measures ANOVA on Ranks: $\chi^2_{0.05, 4}$= 4.400, p = 0.355).

A two way repeated measures ANOVA showed that the degree to which the odourants affected the percent change in distance travelled was influenced by time period within the trial (Figure 2.13; *Ftreatment x time*$_{36, 499}$=1.624, p =0.016; rank transformed). With increasing time, FE effects increased, H3NO effects decreased and AA effects were biphasic. Independently however, odourant treatment and trial time did not affect the percent change in total distance travelled (*Ftreatment*$_{4, 499}$=2.013, p =0.113 and *Ftime*$_{9, 499}$=0.398, p =0.932 respectively). There was no difference in distance travelled for AA, NT or FE treatments vs. EM (Holm-Sidak, p> 0.05). Although both AA and FE odourants had displayed numerically greater changes compared to EM, high variability within treatments prevented distinct differences. H3NO exposure however elicited an increase in distance travelled during minute 1 of odourant exposure as compared to EM (H3NO vs EM: 75.80 ± 31.75% vs. -16.11 ± 16.32%; Holm-Sidak, p=0.0326).

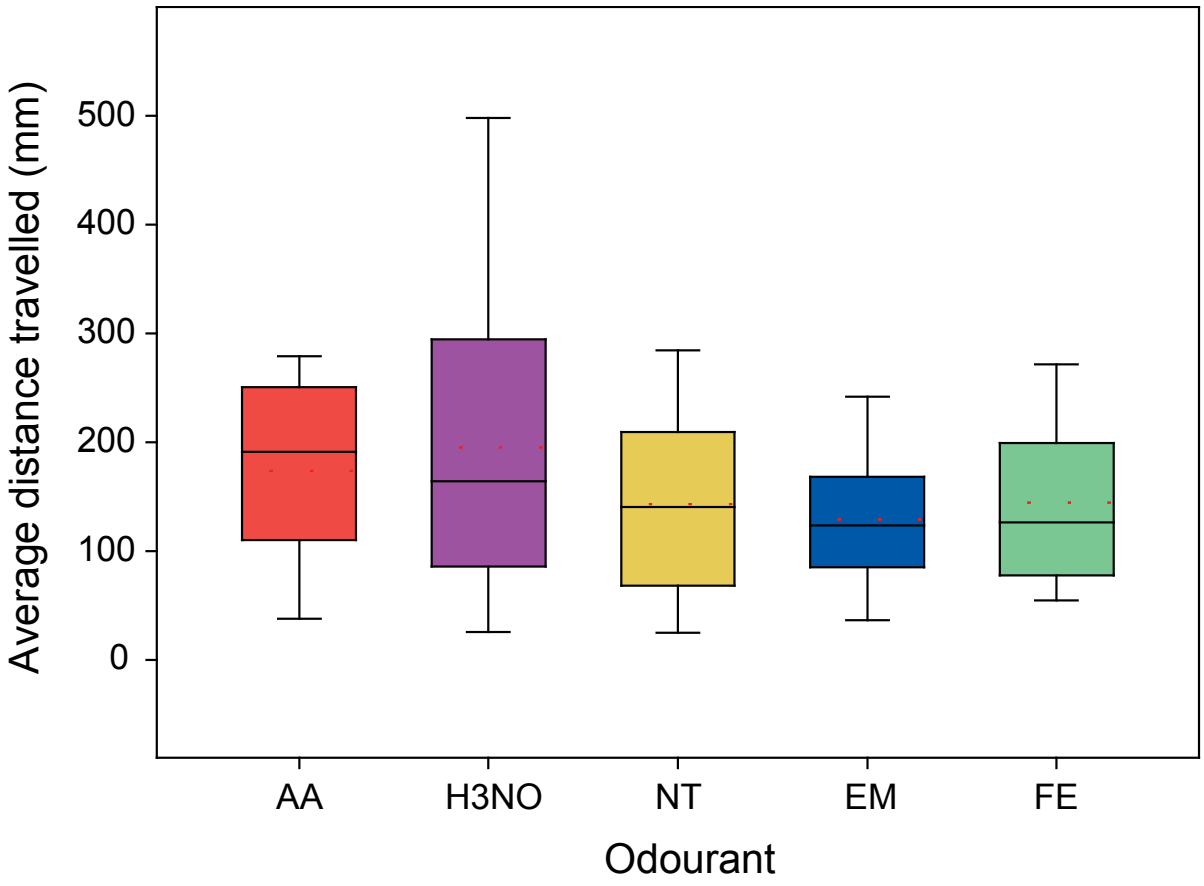


Figure 2.12. Average distance travelled (mm) during baseline activity pre odourant exposure. Box plots represent average distance travelled per minute during five minutes of acquired baseline activity (n=10). Odourants identified are those tested immediately following the baseline activity: amino acid mixture (AA), Hypoxanthine-3-*N*-oxide (H3NO), nucleotide mixture (NT), embryo medium (EM) and food extract (FE). Black lines within boxes represent the median and the whiskers denote minimum and maximum values. Baseline distance travelled does not differ between groups.

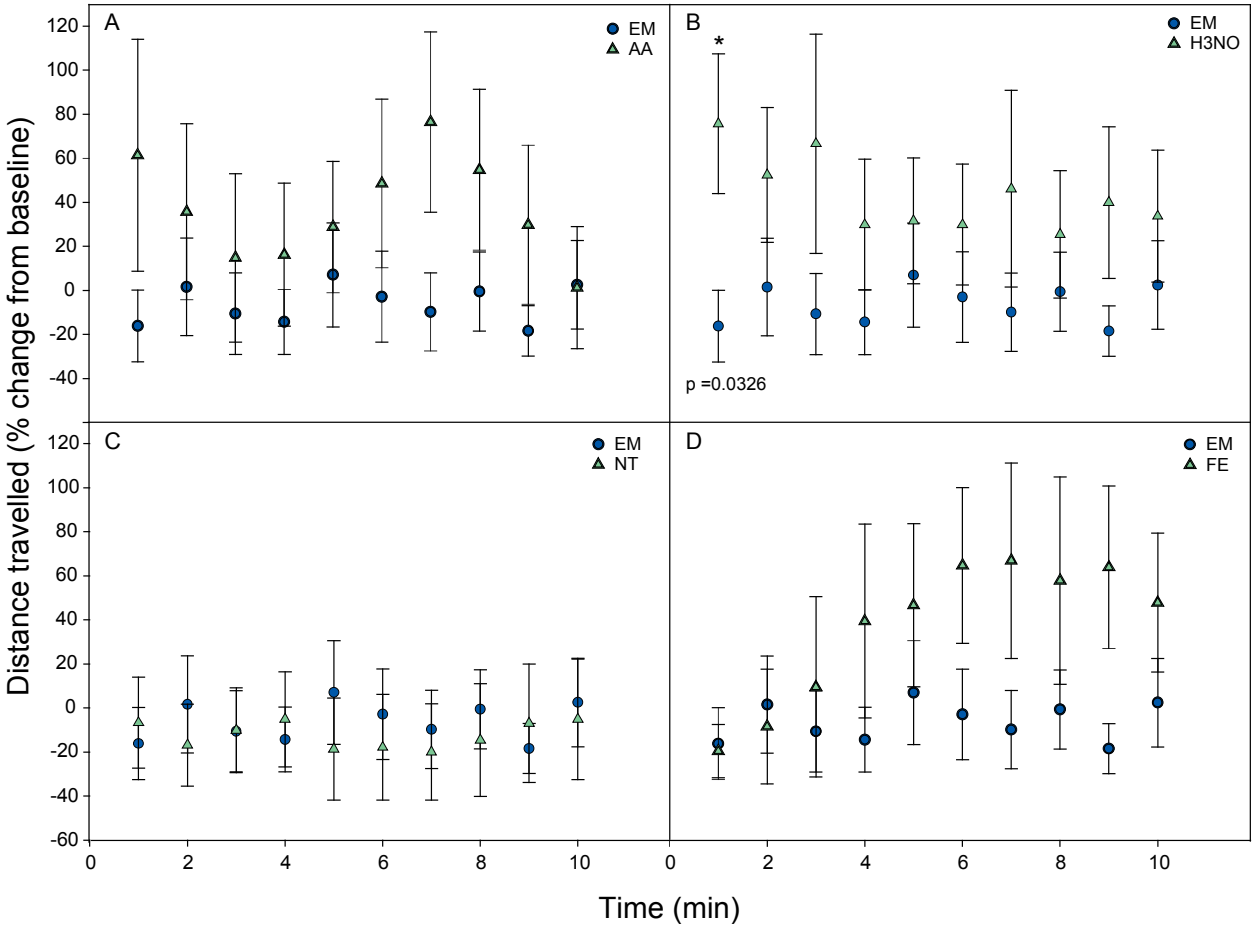


Figure 2.13. Percent change from acclimated distance travelled during odourant exposure. Data represents mean percent change ± SE from individual larval baseline averages (n=10). Positive and negative values indicate increases and decreases from baseline activity respectively. A-D show exposures to control embryo medium (EM) compared to amino acid mixture (AA; $4x10^{-5}$M), hypoxanthine 3-*N*-oxide (H3NO; $5x10^{-9}$M), nucleotide mixture (NT; $4x10^{-5}$M) and food extract (FE; 1:100) respectively. EM data is shown four times in A-D. Asterisk denotes statistical significance.

Time spent in border

Total time (s) spent in the chamber border zone during the five minutes of baseline acclimated activity did not differ between each pre-odourant period (Figure 2.14; Friedman repeated measures ANOVA on Ranks: $\chi^2_{0.05, 4}$= 2.160 p = 0.706). A two way repeated measures ANOVA showed that trial time affected the degree to which odourant treatments altered time spent in the border zone (Figure 2.15; *Ftreatment xtime*$_{40, 549}$=1.501, p =0.030; rank transformed). Odourant treatment and trial time did not independently affect the time each fish spent within the border zone (*Ftreatment*$_{4, 549}$=1.067, p =0.387 and *Ftime*$_{10, 499}$=1.166, p =0.324). Neither EM nor any odourants elicited a change in border zone occupancy compared to baseline (Holm-Sidak, p>0.05). H3NO exposure however, did result in a decrease in border occupancy compared to EM in 6 min of odourant exposure (shown as 7 min in figure; H3NO vs EM: 20.57 ± 5.20s vs. 39.54 ± 6.27s; Holm-Sidak, p=0.0199). Additionally, during the final minute post-odourant addition (10 min, shown as 11 min in figures), both AA and NT treatments caused increases in border zone occupancy vs. EM (AA: 36.01 ± 6.34s and NT: 35.21 ± 7.40s vs. EM: 19.41 ± 5.14s; Holm-Sidak: p=0.0510, p=0.0530).

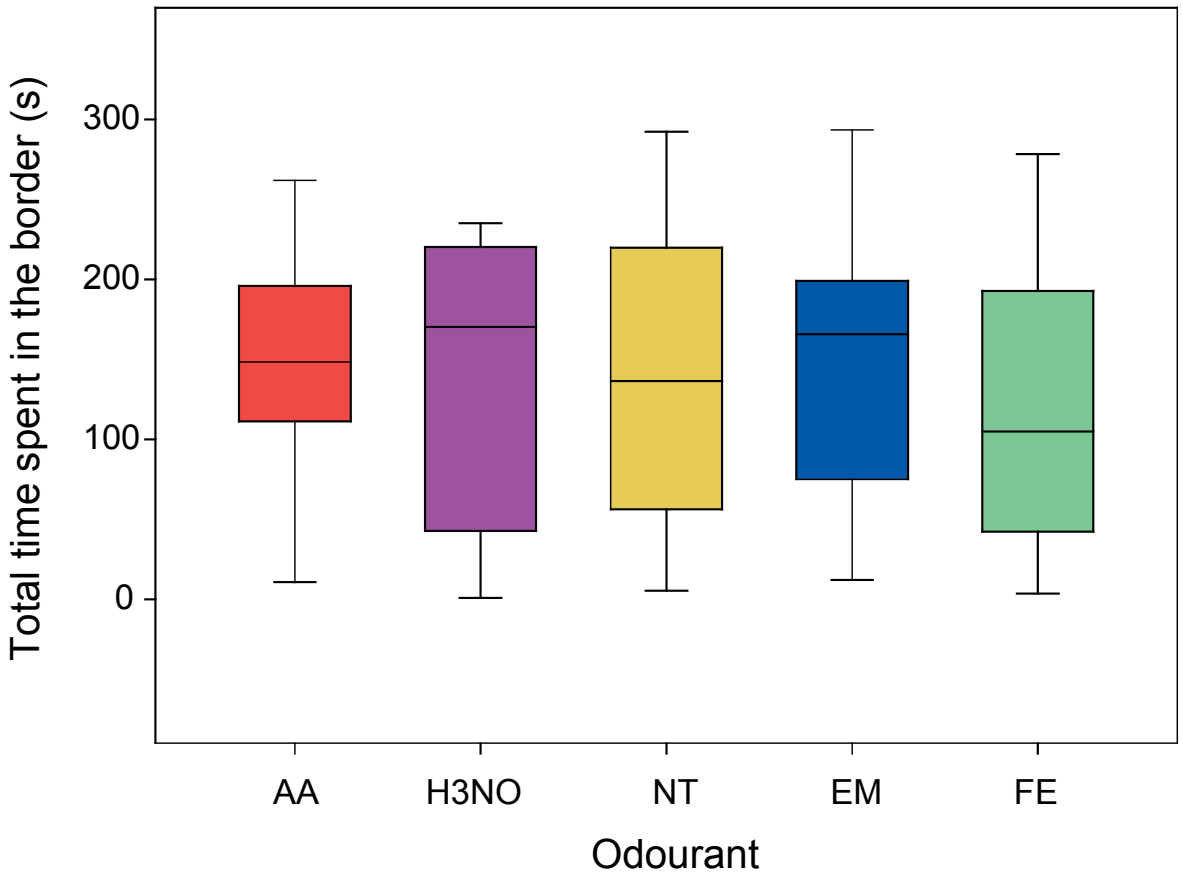


Figure 2.14. Total time spent in border zone during baseline activity pre odourant exposure. Box plots represent total time (s) spent in border zone during five minutes of acquired baseline activity (n=10). Odourants identified are those tested immediately following the baseline activity: amino acid mixture (AA), Hypoxanthine-3-*N*-oxide (H3NO), nucleotide mixture (NT), embryo medium (EM) and food extract (FE). Black lines within boxes represent the median and the whiskers denote minimum and maximum values.

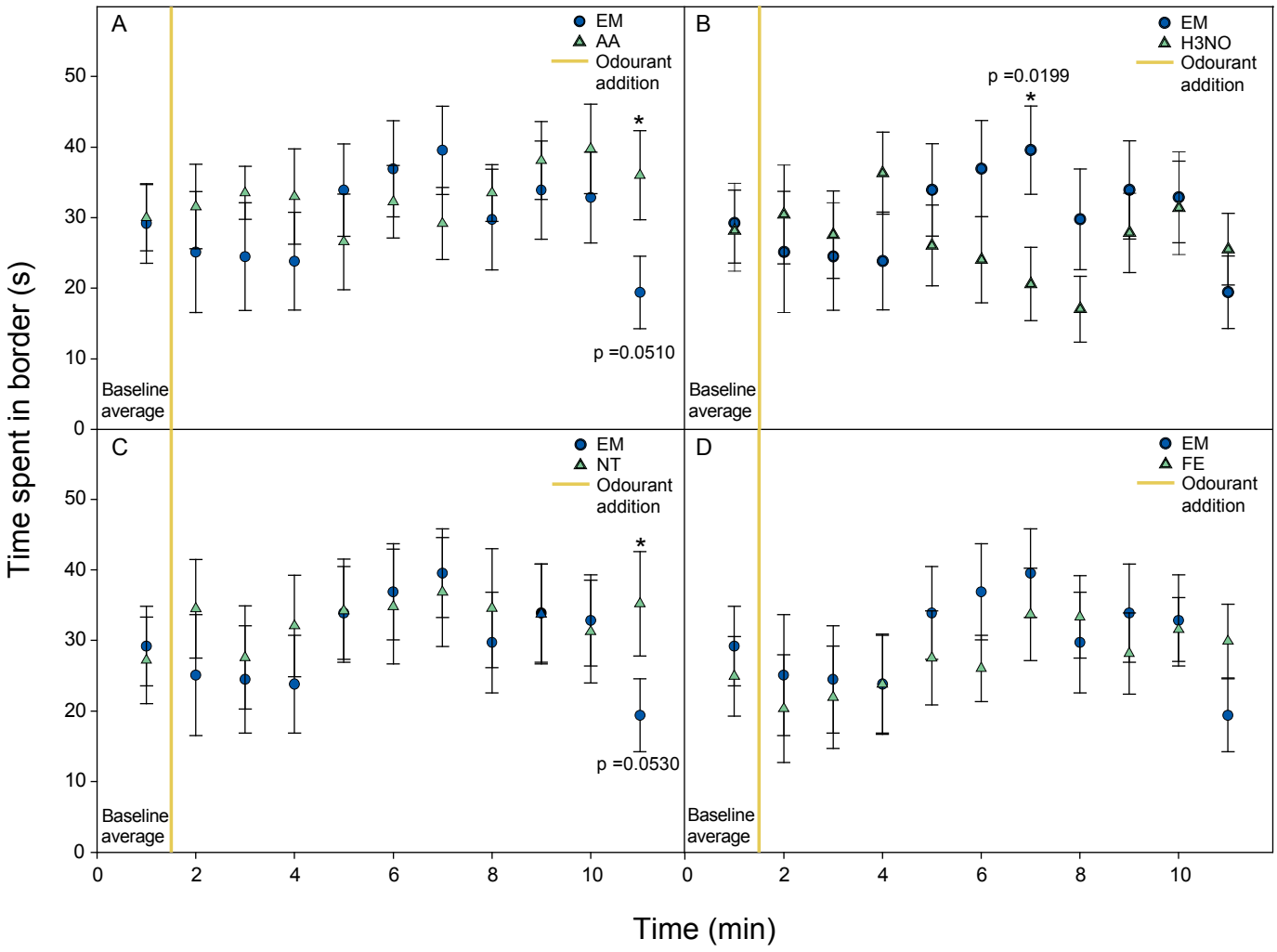


Figure 2.15. Time spent in border zone during odourant exposure. Data represents mean time spent in border zone per minute ± SEM (n=10). Yellow line denotes odourant addition. Data point at minute one represents average baseline time spent in border zone over five minute acquisition period. A-D show exposures to control embryo medium (EM) compared to amino acid mixture (AA; $4x10^{-5}$M), hypoxanthine 3-*N*-oxide (H3NO; $5x10^{-9}$M), nucleotide mixture (NT; $4x10^{-5}$M) and food extract (FE; 1:100) respectively. Asterisk denotes statistical significance between EM and odourant per time point.

Maximum velocity

Maximum velocity (mm/s) during the five minutes of baseline activity did not significantly differ prior to each odourant exposure (Figure 2.16; one way repeated measures ANOVA: $F_{4, 49}$=2.217, p =0.087). Despite not being significant, there was a numeric decrease in max velocity as the trial time increased (pre-odourant maximum velocities: are 238.76 ± 25.1, 178.84 ± 32.57, 157.17 ± 22.20, 140.85 ± 25.19, 144.67 ± 30.52mm/s for AA, H3NO, NT, EM and FE respectively). Pre-odourant maximum velocities were also compared to maximum velocities during and post odourant exposure, the latter referring to trial time post washout (Figure 2.17). A two way repeated measures ANOVA found odourant treatment did affect maximum velocity ($Ftreatment_{2, 149}$=31.752, p <0.001). Trial time also affected maximum velocity as seen through higher values during exposure and lower values during the post exposure windows ($Ftime_{4, 149}$=5.149, p =0.002). Odourant treatment and trial time together influenced the maximum velocity as seen by effective odourants inducing an increase during the exposure window ($Ftreatment\ x\ time_{8, 149}$=2.612, p =0.014). Within the odourant exposure window, AA-evoked maximum velocity (294.30 ± 26.53mm/s) differed from NT (140.18 ± 31.93 mm/s), EM (138.22 ± 30.98mm/s) and FE (194.64 ± 25.11mm/s) (Holm-Sidak, p<0.05). The H3NO-evoked maximum velocity (261.95 ± 30.96 mm/s) was higher than NT and EM (Holm-Sidak, p<0.05). Interestingly, within the exposure period only H3NO evoked an increase in maximum velocity compared to pre exposure values (Holm-Sidak, p<0.05). H3NO-evoked maximum velocity was also different from maximum velocities post exposure (132.23 ± 25.13mm/s), however post exposure maximum velocity was not different from pre exposure values indicating a spike in activity during odourant presence (Holm-Sidak, p<0.05). Maximum velocities for FE were not different across trial time periods (Holm-Sidak, p>0.05). Post exposure maximum velocities for

AA (104.16 ± 30.35mm/s) and EM (54.05 ± 8.51mm/s) were lower than both pre exposure and exposure values, indicating a decrease in maximum velocity compared to baseline as the trial progressed (Holm-Sidak, $p<0.05$). NT post exposure maximum velocity (76.32 ± 23.45mm/s) was also different from pre-exposure baseline values (Holm-Sidak, $p<0.05$). Post exposure maximum velocities did not differ significantly across odourant groups (Holm-Sidak, $p>0.05$).

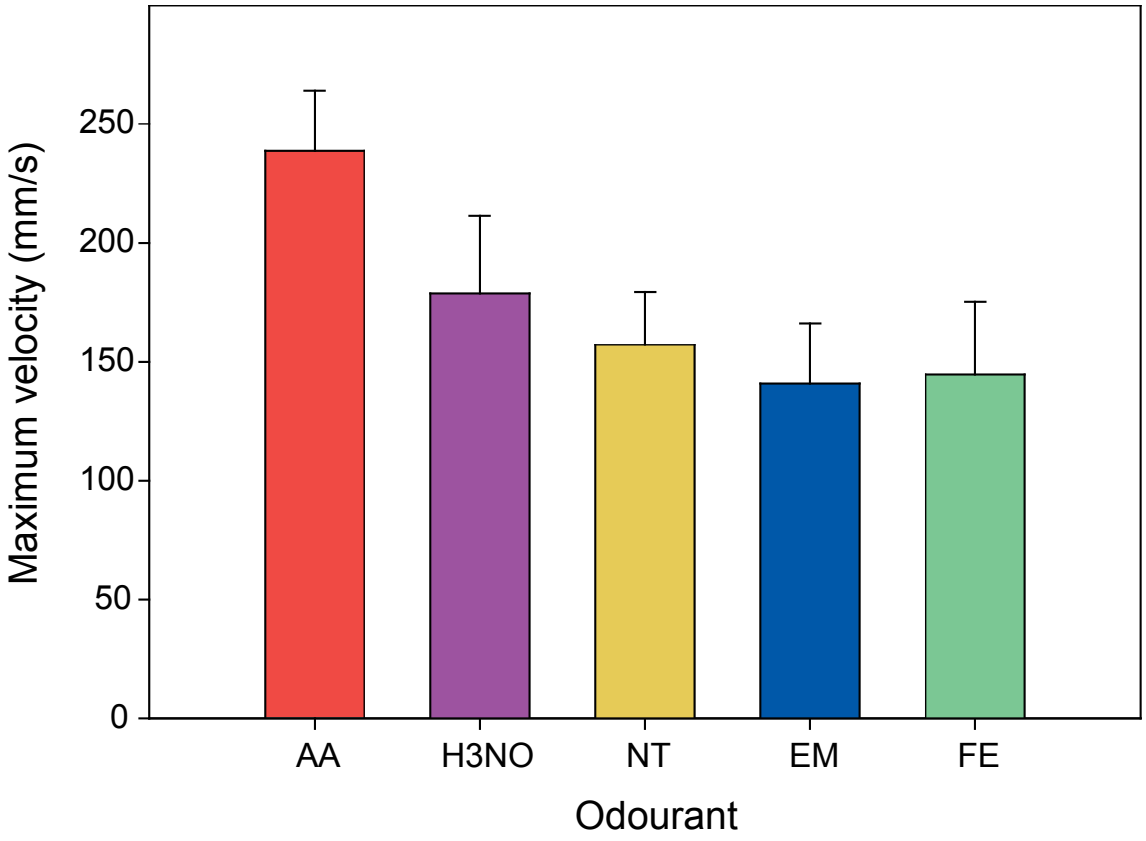


Figure 2.16. Maximum velocities (mm/s) during baseline activity pre odourant exposure. Bars represent average maximum velocity + SEM during five minutes of acquired baseline activity (n=10). Odourants identified are those tested immediately following the baseline activity: amino acid mixture (AA), Hypoxanthine-3-*N*-oxide (H3NO), nucleotide mixture (NT), embryo medium (EM) and food extract (FE).

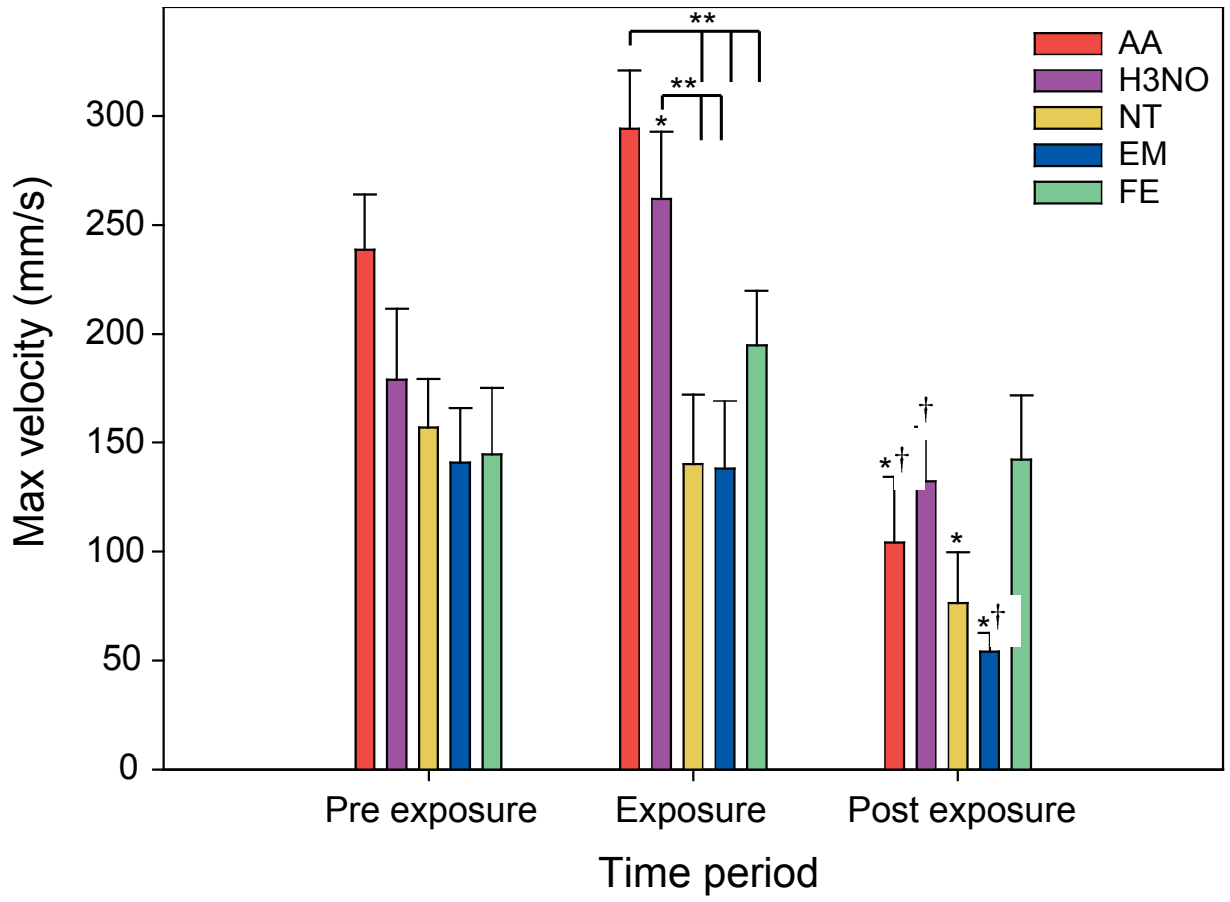


Figure 2.17. Maximum velocities (mm/s) pre, during and post odourant exposure. Bars represent average maximum velocity + SEM during selected time periods. Odourants tested include: control embryo medium (EM), amino acid mixture (AA; $4x10^{-5}$M), hypoxanthine 3-*N*-oxide (H3NO; $5x10^{-9}$M), nucleotide mixture (NT; $4x10^{-5}$M) and food extract (FE; 1:100). Single asterisk denotes significant difference from pre exposure, double asterisk denotes significant difference within exposure period, and dagger denotes difference from exposure period.

Discussion

Ultimately this chapter serves to demonstrate the functionality of two novel devices for testing larval olfactory-evoked behaviour. In developing these assays, new positive controls were identified. Preliminary use of the avoidance-attraction trough revealed larval aversion and neutrality to predicted EE and FE odourants, respectively. Flow-through system experiments characterized the previously unknown larval odourant-evoked alarm responses to H3NO. The methodology and data described represent the foundation for future behavioural studies during fish olfactory development.

Avoidance-attraction trough

As previously discussed, developing a small scale avoidance-attraction trough that operated statically without fluid addition presented an interesting, but necessary challenge to testing larval odourant-evoked behaviours. Initial studies demonstrated that the apparatus can be used to identify robust odourant avoidance-attraction responses. Although certain limitations have been identified in the validation process, the efficacy of the system and potential for future studies overwhelms the minor faults.

Importance and limitations of dye trials

Of primary importance during apparatus prototyping were the food colouring dye trials conducted to obtain a visible representation of the device's fluid dynamics. Dye trials followed exact experimental protocol, including the presence of zebrafish larvae, in order to observe any fluid mixing enhanced by fish movement. Despite the fish movement, zero mixing occurred between the dyes on opposite ends of the apparatus, allowing for determination of consistent odourant vs. non-odourant zones (ROIs). Additionally, there was no diffusion of dye outside

into the middle chamber demonstrating that larvae would not be inadvertently exposed to odourants prior to barrier removal. Differences in molecular weight between odourants and dyes make it important to stress that dye trials are only an approximation of fluid dynamics. While it is certain that odourants will consistently be present within the dye defined ROIs, conceivably odourants could be diffusing past the ROI boundaries. Experimentally, this means that false positives (fish falsely reported in the odourant zone) are not a risk, however false negatives (fish falsely reported outside of odourant zone) are possible. This represents a limitation to this method that can be remedied by a differential analysis discussed in Chapter 3. The potential fault notwithstanding, successful dye trials allowed for further apparatus experimentation.

Fish distribution during control trials

During EM control trials, it is rational that the distribution among the three chambers would be initially skewed towards the middle as all fish begin within the middle chamber. Additionally, although barrier removal is relatively smooth, it does serve as a disruption within the apparatus and likely affects larval movement within the first few measurements. However, as expected, due to identical conditions throughout the apparatus, after a given amount of time, fish move outside the middle chamber and equilibrate among the three areas. This result was essential to apparatus development as it effectively demonstrated that there was no behavioural bias to one area of the apparatus. A side bias would impair the ability to test odourant avoidance or attraction. In future, EM trials should always be conducted in parallel to odourant trials to ensure that no biases have been introduced.

Avoidance-attraction behaviour in control vs. odourant conditions

Observation of equal distribution across the apparatus led to the prediction that the addition of a deterrent or an attractant on either side of the apparatus would cause a shift in distribution. Within the odourant experiments that followed, this expectation, and therefore the apparatus, was supported. Based on previous studies that demonstrate alarm responses to skin extract, or *Schreckstoff*, and avoidance responses (reduced time spent in odourant zone) to cadaverine and other polyamines (carcass-associated) [42, 44, 46, 47], I predicted that zebrafish larvae would respond to EE with avoidance. This was supported as a lower proportion of fish consistently occupied the EE area compared to the non-odourant, EM area. This was likely not a false negative because of the higher proportion of fish present in the opposite side of the apparatus compared to the middle, indicating that most fish had moved as far away as possible from the EE. To date, this is developmentally the earliest observed zebrafish response to a variation of the classical skin extract. It is particularly interesting whether or not this avoidance response could be categorized as innate. Zebrafish olfactory tissue has been found to be odourant responsive as early as 2.5-3 dpf via calcium imaging [11]. Although the majority of non-viable embryos die prior to hatching, because fish are raised in groups, they may be exposed to embryo death post functional development of the olfactory system. As a result, these fish would not arguably be naive to odourants related to expired or damaged tissue and their association with death. Furthermore, larvae already have receptors for odourants specific to this category, such as chondroitin sulfate and cadaverine, shown by these compounds stimulating zebrafish olfactory tissue as early as 6-9 dpf [47]. As with the multiple components of skin extract, exactly which compound(s) the larvae are responding to within EE is unknown. However, now that my experiment has confirmed that EE can be a positive control for larval avoidance-attraction, future

studies can investigate avoidance-attraction responses to individual extract components and tackle the question as to whether these responses are innate or acquired with experience. Interestingly, complete avoidance to EE was not observed and there was consistently a small proportion of fish that did not avoid the extract. Calcium imaging studies paired with behavioural responses could be performed to determine whether EE stimulates the same neurological tissue in both cases. It is possible that the habenula, the higher olfactory neural structure associated with behavioural motivation, is differentially stimulated in these two types of responders [49]. Alternatively, it is possible that the subpopulation that appears to prefer EE was actually exhibiting a fear response and was freezing within the odourant chamber. Future, more intricate analysis of individual fish activity would be necessary to confirm this. Another important observation was that the avoidance response lasted for the duration of the trial. Apart from the potency of the odourant, the extended response may in part be due to the design of the apparatus. Dye trials showed that the relatively static fluid environment allowed for a distinct odourant front. This means that the fish were able to move in and out of the odourant zone and physiologically, they likely did not adapt to the olfactory stimulus.

Based on zebrafish attraction responses to odourant feeding cues and observed larval olfactory bulb stimulation by FE, I predicted that FE would be a potent attractant for 7 dpf fish [11, 28, 38]. In addition to previous findings, it was expected that larvae would be attracted to FE out of the need to find a food source. At 7 dpf the yolk sac which sustained the larva up until this point is depleted, therefore the fish will need to feed and have been observed to feed in other studies at this age [33, 62]. Unexpectedly, the fish were not drawn to the FE; however neither were they deterred, suggesting that FE was a neutral compound. There are several possible reasons behind why FE was not attractive. Firstly, it could be an absence of detection due to the manual scoring

method. Since scoring depends on individual snapshots throughout the trial, it does not take into account the actual duration of time that each fish spends in the odourant vs. non-odourant zone. It also does not examine swimming activity. It would be expected that FE would stimulate food searching behaviour, and therefore fish would be expected to increase exploratory behaviour throughout the apparatus. As the manual scoring method only examined snapshots, this type of behavioural response goes unobserved. This particular limitation and its solution are discussed further in Chapter 3 where a new method of analysis is applied. Secondly, the FE concentration (1:10) was chosen for these validation trials based on its ability to stimulate larval olfactory tissue [11]. However, the concentration is considerably higher than is considered behaviourally relevant and as such for some fish FE may have acted more as a deterrent, countering any positive responders. Some attractive stimuli can become aversive with increasing concentration [73]. Thirdly, the 7 dpf larvae tested are up to this point naïve to olfactory feeding cues. Innate larval feeding has been previously observed, however it was in the context of prey capture where fish were more reliant on visual rather than olfactory cues [62]. It is possible that the fish did not yet have a positive association with the FE *odours* and as such were not attracted. Other genuine *attractive* responses to food related odourants in zebrafish occur in later developmental stages, post introduction to food [28, 37, 38]. Future studies should include repeat exposures to FE to in order determine whether a positive effect can be acquired with increasing familiarity, or if association with actual food is the prerequisite for responses (see Chapter 3). Ultimately, it is an important finding that FE did not elicit a measurable attractive response and likewise future olfactory feeding cues may not be testable using this method.

Dual odourant avoidance-attraction trials were also conducted to address whether fish could be persuaded into making a decision, thereby reducing the number of fish in the middle neutral

chamber. Experiments did result in a lower proportion of fish on average in the middle chamber. EE was still strongly avoided, but proportionally less than when it was alone, suggesting that some fish actually preferred EE over FE. The FE zone also had higher fish occupancy than when singularly tested, but this cannot be distinguished from the effect of EE driving the majority of fish to the opposite side of the apparatus. Overall the experiment only further supported the aversive and inconclusive effects of EE and FE respectively. Nevertheless, the effect of dual odourants motivating a higher proportion of decision making is a technique to be aware of for comparing odourants in future olfactory studies.

Potential for social influence in group testing

Another factor to consider is the potential impact of social influence in group testing within the avoidance-attraction assay. According to Engeszer and colleagues, zebrafish develop shoaling behaviours as early as 12 dpf into development [53]. Due to the early development of shoaling, some larvae may exhibit a tendency to stay together in the apparatus. Theoretically, this concept may work to the benefit or detriment of the method depending on the proportion of weak vs. strong responders. In previous adult olfactory behaviour studies, group size was found to influence responsiveness to L-alanine (feeding cue) [51]. Specifically, among test groups of 1, 2, 4, 6, or 8, a sample size of 4 elicited the greatest response, suggesting that an increased group size is not positively correlated with the number of responders. Additional studies should test different sample sizes in the trough to determine the optimum group number for the assay. Furthermore, the inter-fish distance during avoidance-attraction trials should be measured to determine possible shoaling behaviours.

Flow-through apparatus

The flow-through system was a critical development in odourant testing for individual larvae as fish were acclimated to the water movement and therefore only faced a single stimulus: odourant. Additionally, because of apparatus design, individual fish behaviour in response to multiple odourants and to a control can be taken into account. The experiment described has been an essential test in identifying positive controls for changes in activity and for examining the experimental procedure prior to future investigations (see Appendix A for troubleshooting and operation notes).

Dye trial benefits

Dye trials were conducted to examine fluid dynamics within the flow-through system and were a fundamental step in the development process. The use of dye allowed for correcting the concentration of the odourant delivery solutions to account for chamber dilution. Consequently, the maximum odourant concentration per chamber was known. Dye trials also enabled determination of a washout time point where odourant concentration would no longer be considered significant. This was determined from absorbance data to be 8 minutes post-initiation of odourant delivery. Visually, although lightened in colour, the chambers still appeared to contain a substantial amount of dye, which is attributed to the reflective characteristics of the glass. Absorbance values however, were determined to be a more accurate representation of relevant dye presence. Notably, there can be variations between the custom glass chambers and in order to have equivalent flow rates and washout times, multiple chambers had to be tested and matched for internal fluid dynamics.

Baseline behaviours

The odourant delivery trials were designed such that fish were exposed to different odourants in succession, which raises the question of whether there is sufficient time between exposures for the fish to lose any physiological adaptation to the previous odourant. Previous electrophysiology studies have shown that a two minute recovery window post odourant stimulus is enough time for olfactory sensory neurons to return to baseline [8]. Similarly in larval zebrafish neural wide-field fluorescence microscopy, approximately 3 minutes were allotted between odourant deliveries and effective olfactory tissue stimulation [49]. Therefore physiologically, larvae should have been able to sense the different olfactory stimuli even if successive odourants shared receptor types. Behaviourally, previous studies involving larval zebrafish vary from 3 to 60 minutes for acclimation post transfer, with the most common timeframes being 15-20 minutes [29, 50, 58, 60, 74, 75]. In this experimental design, fish were allowed 20 minutes to acclimate post transfer from a static, group environment to the dynamic, individual test chamber. Between odourant exposures, fish were given 10 minutes post washout, i.e. 10 minutes of EM flow, to return to baseline. Although behavioural re-acclimation was predicted based on published studies, the appropriate time window could only be determined from comparing behaviours during pre-odourant acclimated activity across the trial. No behavioural differences were observed in the selected endpoints during each pre-exposure period, indicating that 10 minutes of re-acclimation between stimuli was sufficient for behavioural recovery. This was incredibly important because fish that were still reacting to the previous stimulus would be likely to have a diminished response to future stimuli. As previously discussed, the test odourants were as follows: AA mix as a food odourant, H3NO as an alarm compound, NT mix as an unknown (appetitive or alarm), EM as a control and FE as feeding

odourant [45]. Odourants were tested in the designated order so that no potential food odourants were tested in succession. It is recognized that this is just one testing order and further experiments with differing odourant sequences should be conducted to establish whether the precursor odourant affects subsequent responses. Although additional experiments are still necessary to further validate the experimental design, because baseline activity did not shift across the trial for this dataset, fish were considered re-acclimated prior to each exposure.

Odourant-evoked changes in total distance travelled

The standard error of the mean was high for the data, as is generally expected for animal behaviour, especially in experiments where sample size is small [50]. To this end, examining changes in each test subject helps limit variability, which was why all statistics were run as repeated measures analysis.

I also attempted to normalize variability in total distance travelled. For each individual fish, data was transformed to percent change from baseline activity to ensure that the endpoint was characterized as stimulus response and not occluded by individual variations in activity level. In this respect, although the absolute value for distance travelled may be high or low, only the difference from the individual's baseline was considered. It was expected that percent changes would be greater (positive or negative) for odourant exposures compared to EM exposure. While this incorporated an important factor, the small sample size still resulted in high individual variation. Further trials may clarify some of the non-significant, but positive trends I observed. Specifically, the observed increase in distance travelled during FE exposure was anticipated based on previous findings that larval activity increases in response to feeding cues [29]. The variability in responses may also be affected by a subpopulation of fish either responding with a decrease in activity (slightly startled) or not responding at all. This is possible because, as

mentioned for the avoidance–attraction trough, 7 dpf fish are still naïve to food-related odourants prior to testing. Also, it is interesting to note that the increases in activity were correlated with the progression of odourant washout, which could be indicative of increased searching behaviour while losing the potential food source. Logically, if the entire test chamber is filled with a food odourant, there is no need to search for it, but as it starts to disappear there may be motivation to forage. Following the same logic explained for FE exposure, the similar increases observed in response to the other feeding cue, AA, may also prove significant with a higher n-value. To isolate whether naivety is indeed a factor, future studies should test whether previous exposure to larval food prior to flow-through testing increases odourant familiarity and subsequent response strength.

The brief increase in distance travelled observed during H3NO exposure represents an important finding. It is the first behavioural response identified to this odourant in larval fish, with all previous testing only having been done in adults[45]. As there is a clear alarm response expressed by jumping and erratic movements in adults, I anticipated fear responses in larvae. Notably, classical fear response can also present as increased immobility or freezing, but H3NO has only elicited increases in activity in both adults [42, 44, 45, 59], and now larvae. Importantly, the observed increase occurred within the first minute of odourant exposure, and this rapid response further supports the compound's role and potency. However since both H3NO and the feeding odourant exposure resulted in increased distance travelled, albeit in different magnitudes and timelines, it was necessary to also examine the other behavioural endpoints to establish odourant 'meaning'.

NT surprisingly did not elicit an observable trend in percent change in total distance travelled as compared to EM exposure. As behaviour did not mimic that observed for the candidate feeding and alarm cues, NT cannot be sorted into either category based solely on distance travelled.

Time spent in the border zone in odourant vs. control exposures

No odourant exposures resulted in significant changes in the amount of time spent in the border zone as compared to average baseline edge preference. Time spent in border area was chosen to measure anxiety-evoked thigmotaxis based on previous studies that observed changes in this behaviour using a static individual well-plate environment [57, 58]. It was unknown whether the flow within the chamber would affect the baseline area of occupancy. It was observed that on average flow-through tested 7 dpf fish spent ~42-50% of their time in the border zone during baseline activity, which is much lower than in static well-plate studies where 5 dpf and 7 dpf larvae spent ~80-90% and ~80% of time respectively in the border [58, 76]. Overall, this demonstrates that within the flow-through environment, fish are less prone to "wall-hugging" behaviour. It is unknown whether this variation is due to fluid dynamics near the walls of the glass chambers or if a flow environment actually serves to reduce anxiety. Some differences in border activity between EM and odourant exposure were observed at unique time points. However, since there were no differences between odourant exposure thigmotaxis compared to baseline averages, the unique differences observed are likely not meaningful. This observation reinforces the importance of comparing exposure activity to pre-stimulus activity in order to ascertain responses and avoid false positives. Since H3NO, an established adult alarm compound, effectively altered other behavioural endpoints, it is surprising that this fear inducing odourant did not instigate increased thigmotaxis. Alternatively, is possible that the particular odourants tested simply do not stimulate thigmotaxis and this endpoint may yet prove valid for

future compounds. This would best be determined by examining larval edge preference within the flow-through in response to established anxiolytic and anxiogenic pharmaceuticals [57, 58].

Changes in maximum velocity during odourant exposure

Maximum velocity, however, did prove to be a usable behavioural metric for the flow-through system as there was a measurable increase during H3NO exposure. The observed increases in velocity were indicative of a dash which is known to be involved in startle or alarm responses [59, 60]. The observed increase in maximum velocity further supports H3NO as an alarm compound for larval zebrafish. Although both AA mix and FE also evoked increases in maximum velocity; there was no difference compared to pre-odourant baseline values. Nevertheless, an increase in maximum velocity (interpreted as fear associated dashing) was not expected for these feeding cues. One possible explanation is the naivety of the test subjects. As previously mentioned the 7 dpf larvae have not encountered a food source and are therefore naïve to food odours. Larval fish being at a vulnerable developmental stage, in their first exposure to unfamiliar odourants, in this case food, they may exhibit a subtle alarm response.

Additionally, although acclimated, pre-exposure maximum velocities did not differ statistically, there was an observable decreasing trend as the trial progressed. This decreased propensity for high velocity dashing may be due either to the fish becoming more acclimated to their environment over time, or a symptom of exhaustion. While the flow rate is lower than other existing flow-through larval devices (2.3ml/min vs. 4ml/min; [29]), after an approximately 2hr trial, it is feasible that fish were reaching physical exhaustion. This is further supported by personal observation that the flow-through motivates fish swimming activity for the majority of the trial. The decline in maximum velocity post exposure must also be considered, especially regarding EM treatment. It may be argued that this was simply an artifact of the reduced post

exposure time bin; however, the decrease in maximum velocity was not consistent across all treatment groups. As such, it could be interpreted that extended exposure to non-stimulating compounds (such as EM) reduced the tendency for erratic movements possibly due to prolonged acclimation time. Each time bin examined was not an equal length; however the reason why maximum velocity had to be examined in terms of odourant presence vs. non-presence was because if I used smaller time intervals the effect would be lost. This was because not all fish would exhibit their dashing behaviour, if they had any, at the exact same time. If dashing present in different time intervals is averaged, there is risk of losing observable effects. Through examining the maximum change within odourant presence, any timing offsets in responses were eliminated. All variations considered, maximum velocity served as an important endpoint and further clarified observed H3NO responses.

Summary

The series of avoidance-attraction experiments ultimately validated the development of a novel design which was effective in testing olfactory-mediated changes in area use in larval zebrafish. Identification of EE as an aversive cue for 7 dpf larvae was critical to the establishment of early odourant-evoked activity. This novel apparatus is unique in its portability, simplicity and rapid experimental execution. With this method, future studies will reliably elucidate the behavioural significance of other candidate odourants. As discussed, the technique itself should in future be further scrutinized concerning additional positive controls, sample size, naivety and alternative analysis methods. While constructed with the central purpose of olfactory avoidance-attraction testing, the avoidance-attraction apparatus may have broader applications. Specifically, the device could foreseeably be used to test water quality, toxicants and their exposure effects, and zebrafish genetic models, all in relation to questions of area use.

Flow-through system results taken together successfully identify H3NO as stimulator of alarm related activity. Exposure resulted in elevated distance travelled and maximum velocity, both of which mirror the erratic movements and activity increases associated with adult zebrafish H3NO induced alarm responses [45]. Of particular interest, was that 7 dpf is now the earliest life stage found to respond to H3NO. Unexpectedly, the NT mixture did not elicit any notable changes in activity, despite being hypothesized to be either an alarm or appetitive substance. This may indicate that the mixture does not evoke behavioural responses measured by the metrics discussed here, or that this particular combination of nucleotides may be an inactive mixture. Future experiments should include both avoidance-attraction testing (see Chapter 3) and alternate combinations of nucleobase compounds to further elucidate their efficacy and association. The lack of activity changes in response to the AA mixture and FE was also unexpected, and as previously discussed this could be symptomatic of the sample size or naivety of the fish. Furthermore, the AA mix specifically does differ in composition compared to previously established mixes and therefore other AA mixtures should be investigated [29]. It is also possible that the behavioural metrics chosen do not effectively measure feeding behaviour. Another indicator of larval zebrafish feeding behaviour is an increase in the frequency of >90° turns [37, 59]. The data should be reanalyzed for this endpoint in order to determine if the fish are responsive to feeding cues within the flow-through device. The default turn quantification in EthoVision XT was not used for this endpoint due to lack of three point tracking system (head, centre and tail). Data obtained via this metric without three point tracking and additional calibration to match manual scoring results is not reliable. Future studies should invest time in calibrating additional EthoVision endpoints such as turn angle and level of mobility to manually scored data for application to flow-through studies. Overall, characterizing stimulus-evoked

changes in activity level for individual larval zebrafish is a new avenue. There remain many other behavioural endpoints to be considered and other odourant groups to be examined in the creation of a positive control library for odour-evoked behaviour. Identification of H3NO and validation of the individualized flow-through method sets the stage for many future investigations that will be critical to understanding larval olfactory development.

The future of the flow-through system

As previously discussed, there are many other experiments that should be conducted to further investigate the potential controls and validate the design of the flow-through apparatus. For example experiments involving different odourant orders, concentrations, single odourant repetitive testing and additional endpoints should all be performed to determine the true effects of the odourants tested.

In a broader scope, the overarching goal of using a flow-through apparatus was to be able to identify behavioural responses despite individual variation. Experimenters could draw comparisons between each fish's individual baseline activity, odourant exposure activity and control (EM) exposure activity. In this sense, the experimenter can then determine how a fish individually responds to positive and negative associated odourants and how the observed behaviour compares to their response to an unknown odourant. Due to the individual variation previously observed in both baseline activity and single stimulus response, it is essential to characterize responses as a deviation from each fish's normal behaviour or risk masking responding subpopulations [50]. For example, in response to a fear-inducing odourant, a fish could either increase activity (dash), decrease activity (freeze), or exhibit a pattern of both, all of which are normal responses, but may cancel each other out in robust group analyses. The solution to this issue is the application of a unique method called behavioural phenotyping which

identifies groups with different responses [50]. The flow-through device is optimally designed for this type of analysis because of its potential to obtain a behavioural repertoire (responses to several different odourant classes) per individual fish. While this will be critical to the progressive understanding of odourant-evoked behaviour in fish, this apparatus has additional potential for applied sciences. Zebrafish have become important models for high-throughput toxicant and pharmaceutical testing, both of which often use changes in activity or behaviour as endpoints [77, 78].

The flow-through in combination with behavioural phenotyping would be valuable in determining toxicant or pharmaceutical effects in different subpopulations. This will prove particularly critical to preliminary drug testing. Identifying subpopulation side effects to medications prior to human consumption would be invaluable in preventing serious health consequences due to unanticipated individual variation. Unfortunately, sample size in this data set is too small for behavioural phenotyping, but future studies should take advantage of the experimental design and positive controls identified to advance this objective.

Conclusion

Both the avoidance-attraction and the flow-through apparatuses were designed out of the need to develop reliable testing devices for larval zebrafish olfactory-evoked behaviour. Each device was created to address a particular question. The avoidance-attraction trough asks whether the compound is a deterrent or an attractant, while the flow-through asks: does this compound elicit a change in activity? Not all odourants will effectively alter both categories of behaviour, therefore when investigating novel compounds it is important to ask both questions. In order to do so, the necessary equipment had to be made available. In addition to the lack of standardized commercially available equipment, positive controls for either behaviour had yet to be clearly

established for zebrafish larvae. Through the series of experiments described, the novel equipment and methods have now been validated and two positive controls, one per method, have been identified. Interestingly enough, for both apparatuses the effective odourant was a potent alarm compound, and surprisingly, food related odours did not significantly alter behaviour raising further questions as to possible innate vs. learned responses to novel olfactory stimuli. Successful validation of this equipment and the associated positive controls has now created a foundation for future experiments to construct a comprehensive understanding of zebrafish olfactory-evoked behaviour during early development. As behavioural responses are the manifestation of interpreted olfactory input, they are crucial to understanding odourant significance to the animal. In doing so, we can further distinguish chemical components capable of stimulating olfactory tissue, compared to those that are necessary to provoke a behavioural response.

Chapter 3: Zebrafish behavioural responses to nucleobase compounds

Introduction

Composition of nucleobase odourants

Nucleotides have long been identified as a class of neurologically operative odourants in fish, or in other words, nucleotide exposure stimulates olfactory neural activity [10, 11, 79]. However, prior to the present study, there was little information on both the behavioural significance of these odourants in fish, as well as the significance of differences in nucleobase chemical structure. Nucleotides are simply one variation of nucleobase compounds which are generally described as biological structures with nitrogen containing rings. These rings can be singular (pyrimidine) or doubled (purine). While the nucleobase forms the foundational building block for these structures, there are chemical additions that serve to modify the bases. The addition of sugar groups, ribose or deoxyribose rings, to nucleobases via a glycosidic bond forms nucleoside structures. Addition of phosphate groups to the sugar ring of a nucleoside forms the nucleotide structure. Nucleotides as a group include nucleoside mono-, di-, and triphosphates. In summary, nucleobase compounds can include solo nucleobases, nucleosides and nucleotides, the latter having variable levels of phosphorylation.

Neurological evidence of odourant efficacy

Despite nucleotides being accepted as odourants in fish, there are few electrophysiological studies on nucleobase compound stimulation of fish olfactory tissue. Detection of a nucleotide mixture (adenosine-5'-triphosphate, inosine-5'-monophosphate and inosine -5'-triphosphate) in the channel catfish (*Ictalurus punctatus*) has been determined via single olfactory bulb neuron recordings[10]. Results indicated a spatial organization of the olfactory bulb, with specific

regions (dorsal and caudolateral) being responsive to nucleotides. For zebrafish specifically, it has been demonstrated that nucleotides (adenosine-5'-triphosphate and inosine-5'-monophosphate) can induce olfactory neuronal signaling as early as 3 dpf [11] . Notably, while several nucleotides have been tested as olfactory neural stimulants, other nucleobase compounds have not. Even studies in other species such as the spiny lobster (*Panulirus argus*), have focused on phosphate containing compounds[65]. Preliminary electro-olfactography work conducted on goldfish (*Carassius auratus auratus*) showed that several non-nucleotide nucleobase compounds such as adenosine, guanosine, adenine and hypoxanthine did stimulate the olfactory epithelium (data not shown; Blunt, unpublished). The neurophysiological evidence of olfactory stimulation in fish, including larval zebrafish, supported my investigation into nucleobase compounds as odourants.

Behavioural responses to nucleobase compounds

Insects

In several hematophagous insect species including bed bugs (*Cimex lectularius* L.) and the kissing bug (*Rhodnius prolixus*), nucleotides have been identified as potent initiators of gorging behaviour [64, 80, 81]. In both species, adenosine-5'-triphosphate has been identified as the most potent feeding stimulant, with compound effects diminishing with reduction of phosphate groups and other deviations from the adenosine-5'-triphosphate structure by changes in the nucleobase identity [64, 80, 81]. Interestingly, these findings are supported by trends observed in nucleotide stimulation of spiny lobster olfactory cells [65].

Fish

Perhaps due to the evidence of nucleotides as feeding stimulants in insects, it is generally assumed that they have a similar effect in fish species. However, among the studies that have been conducted there appears to be conflicting evidence between species and a limited variety of nucleobase compounds tested. A nucleotide mixture of just the adenine family (adenosine- tri, di and monophosphate and inosine-monophosphate) induced feeding (flavoured agar ingestion) in adult rainbow trout (*Salmo gairdneri* R.), but not in Atlantic salmon parr (*Salmo salar*) [35]. While Mearns and colleagues classified their nucleotide mixture as a feeding deterrent for the salmon, they did not investigate any other behavioural endpoints or any other combinations of nucleobase compounds [35]. Inosine-monophosphate tested on its own also elicited feeding behaviour in yellowtail, turbot, jack mackerel and marbled rockfish, while inosine alone stimulated feeding in marbled rockfish, tilapia and turbot [82]. Miyasaki and Harada were far more exhaustive in their nucleobase list when testing black abalone (*Haliotis discus*) and the oriental weatherfish (*Misgurnus anguillicaudatus*), however they again observed differences in odourant efficacy between species and only quantified feeding behaviour [39]. Within their study, Miyasaki and Harada also relay that most odourants evoke an attraction response *or* stimulate activity, but do not evoke both types of behavioural responses. This further supports the idea that a nucleobase compound deemed inactive in one behavioural assay may be found potent in a different experiment. Notably, none of the aforementioned studies that examined feeding behaviour considered examining negative associated responses such as fear or avoidance. The synthetic purine ring H3NO is a potent alarm cue in several fish species including zebrafish [41-43, 45, 63]. Interestingly, rainbow trout (*Oncorhynchus mykiss*) do not exhibit an alarm response to H3NO [63] which further emphasizes the variations in olfactory

systems and evoked behaviours between species. While some experts have argued that the nitrogen-oxide functional group is essential in evoking an alarm response, since some nucleotide mixtures have acted as feeding deterrents in different species, it is possible that nucleobase compounds other than H3NO can also evoke alarm or avoidance behaviours [35, 41]. Within zebrafish larvae, the focus organism for this study, it has been previously determined that adenosine evokes variable, uncharacterized changes in activity [50]. To determine negative or positive odourant associations (avoidance or attraction respectively) to nucleobase compounds, this study applied avoidance-attraction experiments as described in Chapter 2.

Existence of nucleobase compounds in nature

When considering where nucleobase compounds might exist in nature, the debate between alarm vs. appetitive cues is not clarified. Since nucleobase compounds are biological structures, they conceivably come from any biotic source in the forms of DNA, RNA, or essential coenzymes. These compounds may be excreted naturally or may be released involuntarily through damaged or decaying matter. Previous studies have isolated nucleotides from many animal tissues including fish and shrimp extracts and rabbit blood [35, 80, 82]. Depending on the source, the detector may interpret the odourants as a potential food source or as a warning of predator presence. Further complicating the idea, the interpretation may depend not only on the odourant source, but also on the physical characteristics, such as age and size, and the behavioural nature, such as bold or shy, of the detector. For example, what may be a food source to an adult fish, may also be a predator of larvae.

Sources of response variation due to zebrafish age

Although it has been previously established that the zebrafish olfactory bulb can be stimulated by odours at ~3 dpf [11], it is important to recognize that as the larvae continue to develop, their functionality of the olfactory system greatly increases. First, it has been noted that odourant receptor expression increases progressively with age [31, 83]. Second, not all receptors have the same expression onset nor do they increase at the same rate, therefore receptor repertoire generally increases, but fluctuates with age [30, 31]. Calcium imaging has corroborated these findings as greater odourant induced olfactory bulb activity is observed at 5 vs. 3 dpf [11]. The variation within the olfactory epithelium during development suggests the possibility for differences in detection and subsequent behavioural responses between ages. As discussed in Chapter 2, behavioural responses may also differ between ages because as the fish develop they have corresponding increases in baseline activity, physical capacity for movement and need for food [32, 33]. Behavioural responses may also be affected by the individual's familiarity with the odourant. Zebrafish can be conditioned to neutral chemical cues using positive reinforcement (food) and have also demonstrated olfactory memory via kin odourant recognition [37, 84, 85]. As such, it would be interesting to compare responses to nucleobase compounds between naive and familiar larvae.

Research questions and hypothesis

Consideration of the above background regarding nucleobase-evoked behaviour has led to the following research questions and hypotheses that are central to this chapter:

1) **Do nucleobase compounds evoke olfactory behaviours in zebrafish?**

It is hypothesized that nucleobase compounds will evoke olfactory behaviours due to their ability to stimulate neurons and to induce activity in fish of different ages and animals of different species.

2) **Does the olfactory response depend on nucleobase structure?**

   Based on behavioural results in several species, it is expected that compounds containing phosphate(s) will elicit an attraction response, while solo nucleobase rings will provoke an avoidance response.

3) **Do olfactory-evoked behaviours vary with age?**

   Due to the increased mobility, odourant receptivity, and physiological need for food, it is expected that responses, especially attraction, will be more prominent with age.

4) **Do repeated exposures diminish or strengthen behavioural responses?**

   It is expected that repeated odourant exposures without adverse associations will increase compound familiarity and therefore reduce any stimulus avoidance.

The research questions listed above were investigated using an adult zebrafish behavioural apparatus and the larval avoidance-attraction chamber described in Chapter 2.

Materials and methods

Animal use

Adult AB zebrafish were reared in the University of Alberta aquatics facility. Fish were moved to a stand-alone flow through tank system maintained at 28°C and under a 14:10 light: dark cycle. Fish were fed twice daily and were acclimated to the new system for two weeks prior to behavioural testing. Fish were allowed a minimum of 48hrs to recover between behaviour trials. Larval zebrafish were obtained and reared following protocols described in Chapter 2.

Odourants

The following larval nucleobase compounds were tested at $1x10^{-5}$M concentrations and obtained from Sigma-Aldrich (ON, Canada): Adenine, adenosine, adenosine-5′-monophosphate monohydrate (AMP), Adenosine-5'-triphosphate disodium salt hydrate (ATP), guanosine-5′-monophosphate disodium salt hydrate (GMP), guanosine, cytidine-5′-monophosphate disodium salt (CMP), uridine-5′-monophosphate disodium salt (UMP), Inosine-5'-monophosphate disodium salt hydrate (IMP) and hypoxanthine. Stock solutions for nucleobase compounds were made as $1x10^{-3}$M in EM, pH adjusted to 7.20± 0.05 and stored at -20°C prior to testing (see Chapter 2 for EM recipe). The NT mixture (AMP, CMP, UMP, GMP), H3NO and FE were prepared as described previously and tested at final concentrations of $1x10^{-5}$M, $5x10^{-9}$M and 1:100 respectively (See Chapter 2 Materials and methods).

Adult odourant stock solutions (L-alanine and adenosine; Sigma-Aldrich) were prepared using MilliQ purified water and serially diluted fish system water (adults) to the desired exposure concentrations. Test solutions were made fresh weekly and stored at 4°C.

Larval test apparatus and procedure

Zebrafish larvae ages 5, 6 and 7 dpf were tested for avoidance –attraction responses to nucleobase compounds using the avoidance-attraction trough. Specific operation notes are outlined in Chapter 2 methods (n=8-10 trials per odourant group). Trials were conducted in 3 separate odourant groups; the daily order of the test solutions was randomized. Set 1: adenine, adenosine, AMP, ATP, guanosine, GMP and EM; Set 2: CMP, UMP, IMP, hypoxanthine, H3NO, NT mix and EM; and Set 3: Repeat trials EM, AMP and FE. Repeat trials followed the same avoidance-attraction procedure, however the same group of fish was tested at 5, 6 and 7 dpf with the same odourant.

Larval behaviour analysis and statistics

Manual scoring and statistical analysis of odourant Set 1 were conducted as described in Chapter 2. Distributions used were based on those that best fit control trials (left: middle: right as 1/3:1/3:1/3, 2/5:1/5:2/5 or 4.5/10:1/5:4.5/10). Data is presented as mean percent fish occupancy ± SEM (%). For odourant trials, data was analyzed as odourant vs. non-odourant zones compared to the corresponding control proportions (example: 1/3:2/3 odourant vs. non-odourant). All odourant sets, including Set 1 were analyzed using videograms (see Appendix B for sample videogram images). Videos obtained from avoidance-attraction trials were converted to .AVI format with a frame rate of 2 frames per second using VirtualDub (General Public License). AVI files were then converted to videograms in ImageJ (National Institutes of Health, USA) using the protocol outlined by Wyeth and colleagues with several standard adjustments [86] . Average substacks were created by using every 10^{th} frame between frame 100 and the last frame (rounded to the nearest hundred). The mean background image was imported into Adobe Photoshop CS4 (Adobe Systems, USA) and the clone stamp tool was used to eliminate stationary fish from the

background image. The edited mean background image was used in videogram creation to account for fish presence despite lack of movement. Summed slices were used to create videograms per designated time bins. Due to both the lack of significance found in previous analysis of minute 1 and the poor videogram quality due to water disturbance immediately following barrier removal, minute 1 was not analyzed. Videograms were instead made for the time intervals 1-2, 2-3, 3-4, 4-5, 5-7 and 7-10 min. Notably, slight adjustment in camera focus resulted in excessive glare or disrupted frames within the videogram. Disrupted frames were deleted from the videograms to maintain integrity of the data. A maximum threshold of 20% was set for frame deletion within a time bin. Time bins requiring more than 20% frame removal were discarded. As a result, some time bins have a lesser n-value than the total number of trials conducted per individual odourant. Specific points of glare that were not removed by subtraction of the mean image were first verified as image noise and then removed in ImageJ. To visualize tracks, look up table (LUT) was changed to 'Fire'. On the mean background image per trial, an apparatus region of interest (ROI) was made using the polygon tool to draw a shape along the inner edge of the apparatus. Both the length of the odourant side and the coordinates of each corner of the ROI were measured for future calculations. The created ROI was applied to each of the videograms. The x-value for centre of mass within the ROI was obtained for each videogram. The ROI coordinates were used to determine the x-axis value for the apparatus centre in each trial. The shift in fish occupation was calculated as the difference between the measured videogram centre of mass and the apparatus centre. Shifts were converted to millimetre (mm) scale using the actual vs. videogram measured length of the apparatus side. In EM trials positive and negative values indicate shifts to the right and left respectively. In odourant trials, values were corrected to reflect movement in response to odourant side therefore positive and negative

values reflect movement towards and away from odourant side respectively. Data is presented as mean centre of mass shift ± SEM (mm).

Statistical analysis of obtained centre of mass data was performed using two way repeated measures ANOVAs, with Holm-Sidak post-hoc tests. In general, significance is identified as $p \leq 0.05$ unless otherwise noted. Other relevant p-values are listed (<0.10). Behavioural data in larval zebrafish studies can have significant variation as noted in a previous study, therefore an α threshold of 0.10 is considered reasonable [50]. Datasets that violated normality were rank transformed prior to parametric statistical analysis as per Conover's suggestions [72]. Odourant set 2 6 and 7 dpf EM controls displayed on observable bias to the right side of the avoidance-attraction apparatus. As a result, 6 and 7 dpf odourant trials from set 2 were compared to EM controls from set 1 where no bias was present. Values that statistically showed a bias to the right side (two way repeated measures ANOVA) were corrected for this bias prior to comparison to set 1 EM controls. Success of bias correction was tested by repeating the two way repeated measures ANOVA. The correction factor (CF) was defined as follows:

$$CF = \text{Mean EM shift odourant set 1} / \text{Mean EM shift odourant set 2}$$

Repeat trials suffered the same observable right side bias; however no available non biased control set was available for data correction. Results should be interpreted with caution.

Adult test apparatus and procedure

Adults were exposed to test odourants using an established static induction method [42, 45]. Glass test tanks were constructed based on the effective gravity fed designs from other zebrafish olfactory studies [42, 45]. Adults were fasted for 24hrs to enhance potential motivation for feeding responses. Fish were individually placed in L tanks in a dark enclosure and acclimated

for 30 minutes prior to odourant induction. Darkness was maintained to limit visual stimuli from the experimenter or other fish. Tanks were set up so that four fish could be tested simultaneously. Five mL of odourant solution was gravity fed into the tank at surface level to minimize water disturbance (~0.06% volume change). Fish activity was recorded using infrared cameras and videos (5 minutes pre stimulus and 5 minutes post stimulus) were tracked and analyzed using EthoVision XT v.8.5 (Noldus, NE) and SigmaPlot (Systat; CA, USA). Test odourants included adenosine ($1x10^{-5}$M), L-alanine ($1x10^{-4}$M) and system water as a control (n=10 per odourant group).

Adult behaviour analysis and statistics

Infrared videos from adult behavioural videos were tracked under dynamic subtraction settings (~15samples/second) in EthoVision XT v.8.5 (Figure 3.1). Behavioural endpoints examined time (s) spent in bottom half of the tank and velocity (cm/s). Fish positional data for two minutes prior and post odourant exposure was the focus due to the expected immediacy of olfactory responses [42].Aligning the maximum increases in bottom dwelling compared to pre exposure activity per individual fish (spike alignment), was determined by comparing the average time spent in the bottom ½ of the tank (per 30s bins) two minutes before odourant exposure to the maximum % change value for each fish during the two minutes post exposure. Velocity and time spent in the bottom ½ of the tank were analyzed using two way repeated measures ANOVAs and Holm-Sidak post hoc tests where appropriate. Spike alignment data was analyzed using a two way ANOVA with a Tukey post hoc test. Unless otherwise noted, significance was determined by $p< 0.05$.

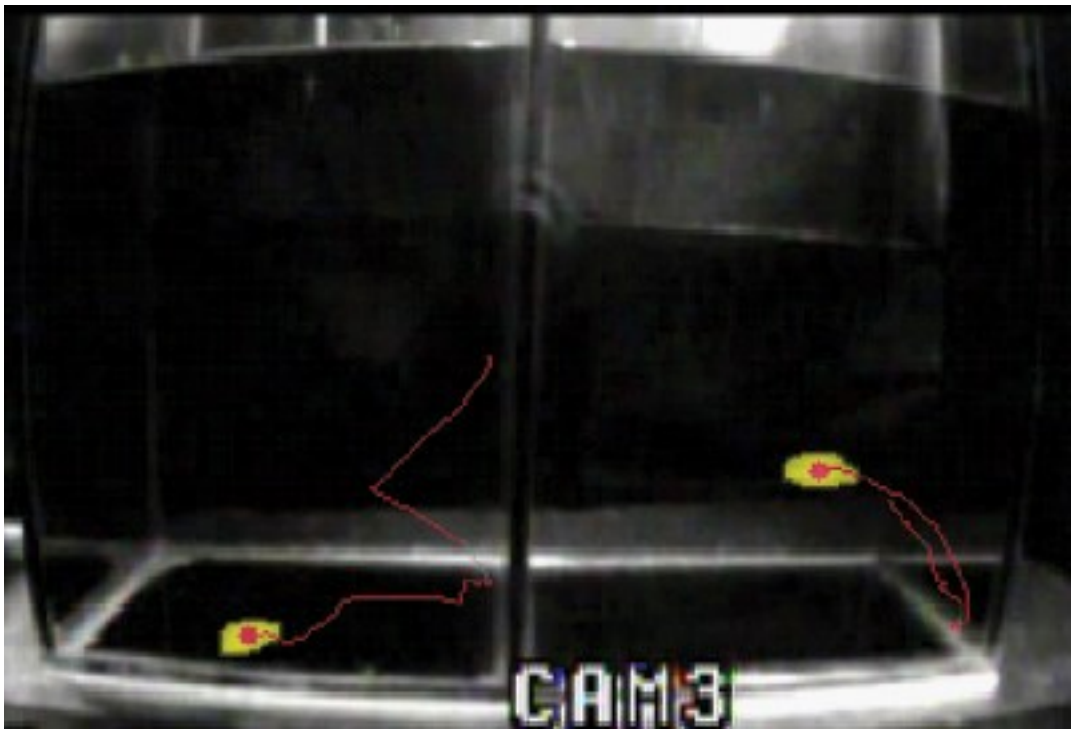


Figure 3.1. Sample image of adult zebrafish video tracking in EthoVision XT. Red marker identifies centre point of the fish.

Results

Adult zebrafish behavioural respond to adenosine

Adult zebrafish showed no change in average velocity following exposure to the adenosine, water or L-alanine (Figure 3.2; two way repeated measures ANOVA: $Ftreatment_{2, 599}$=0.182, p =0.835; Holm-Sidak, p>0.05; n=10 individuals per treatment group). However, fish did display an increase in bottom dwelling activity, quantified as time spent in the bottom ½ of the tank, after both adenosine (0.5-1 min post exposure) and L-alanine (1-1.5 min post exposure) addition (Figure 3.3; two way repeated measures ANOVA; $Ftreatment_{2, 239}$=2.304, p =0.119; $Ftime_{7, 239}$=2.768, p =0.009; $Ftreatment\ x\ time_{14, 239}$ =1.804, p =0.041, Holm-Sidak p<0.050). Spike analysis, further clarified the increases in bottom dwelling behaviour (Figure 3.4; two way ANOVA; $Ftreatment_{2, 59}$=4.261, p =0.019; $Ftime_{1, 59}$=22.636, p <0.001; $Ftreatment\ x\ time_{2, 59}$ =0.932, p =0.400, Tukey p<0.05). Bottom dwelling increased post adenosine exposure (3.3 ± 1.4s vs. 12.6 ± 1.8s) and L-alanine exposure (8.3 ± 1.6s vs. 22.0 ± 2.6s), but not water (9.8 ± 2.0s vs. 16.5 ±2.8s). Additionally, there was no difference in pre exposure activity across treatments (Tukey, p>0.05). L-alanine had increased post exposure activity compared to adenosine (Tukey, p<0.05).

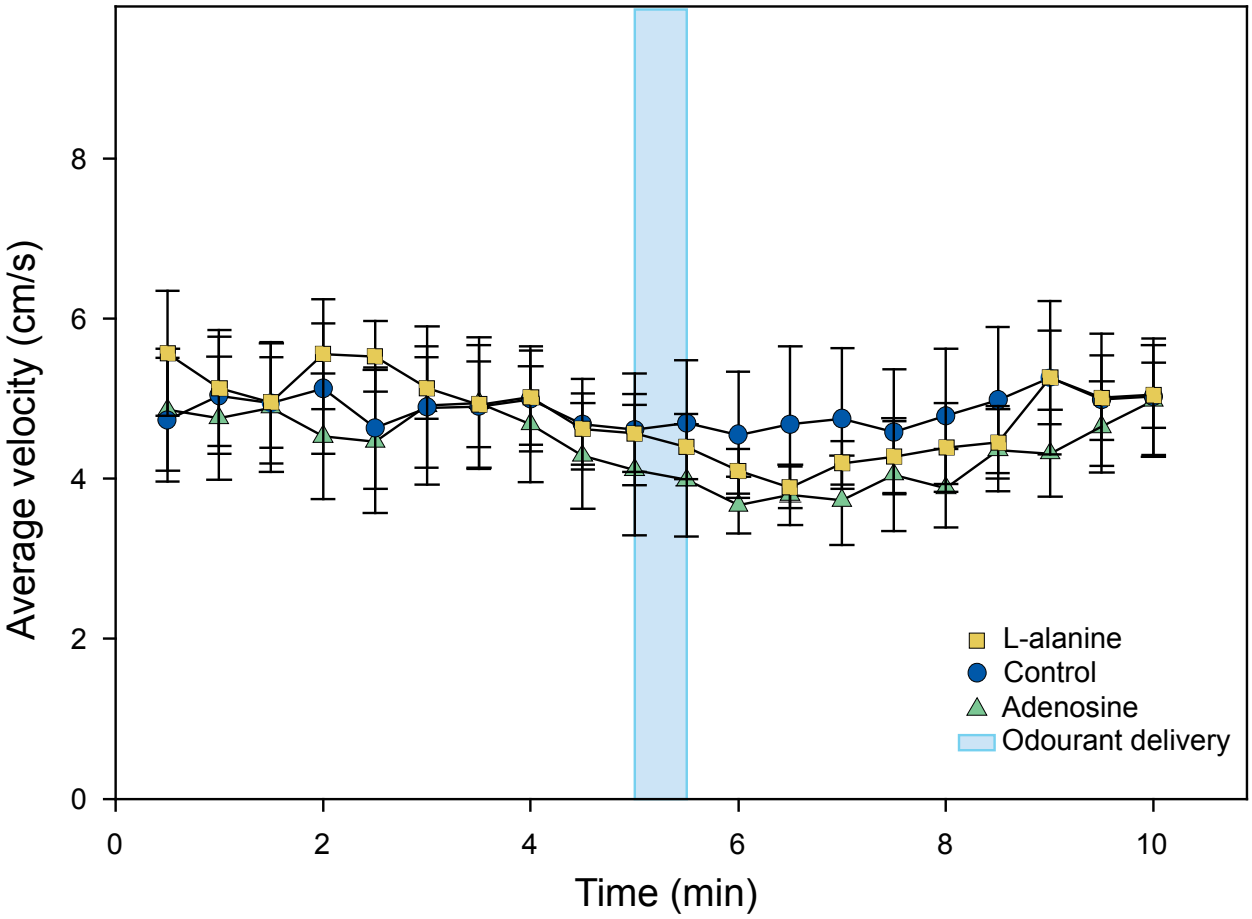


Figure 3.2. Adult zebrafish average velocity (cm/s) pre and post odourant exposure. Fish were exposed to $1x10^{-5}$M adenosine, $1x10^{-4}$M L-alanine or fish system water (n=10 per treatment). Velocity data is presented in 30s bins (mean ± SEM.) Blue segment denotes odourant addition window.

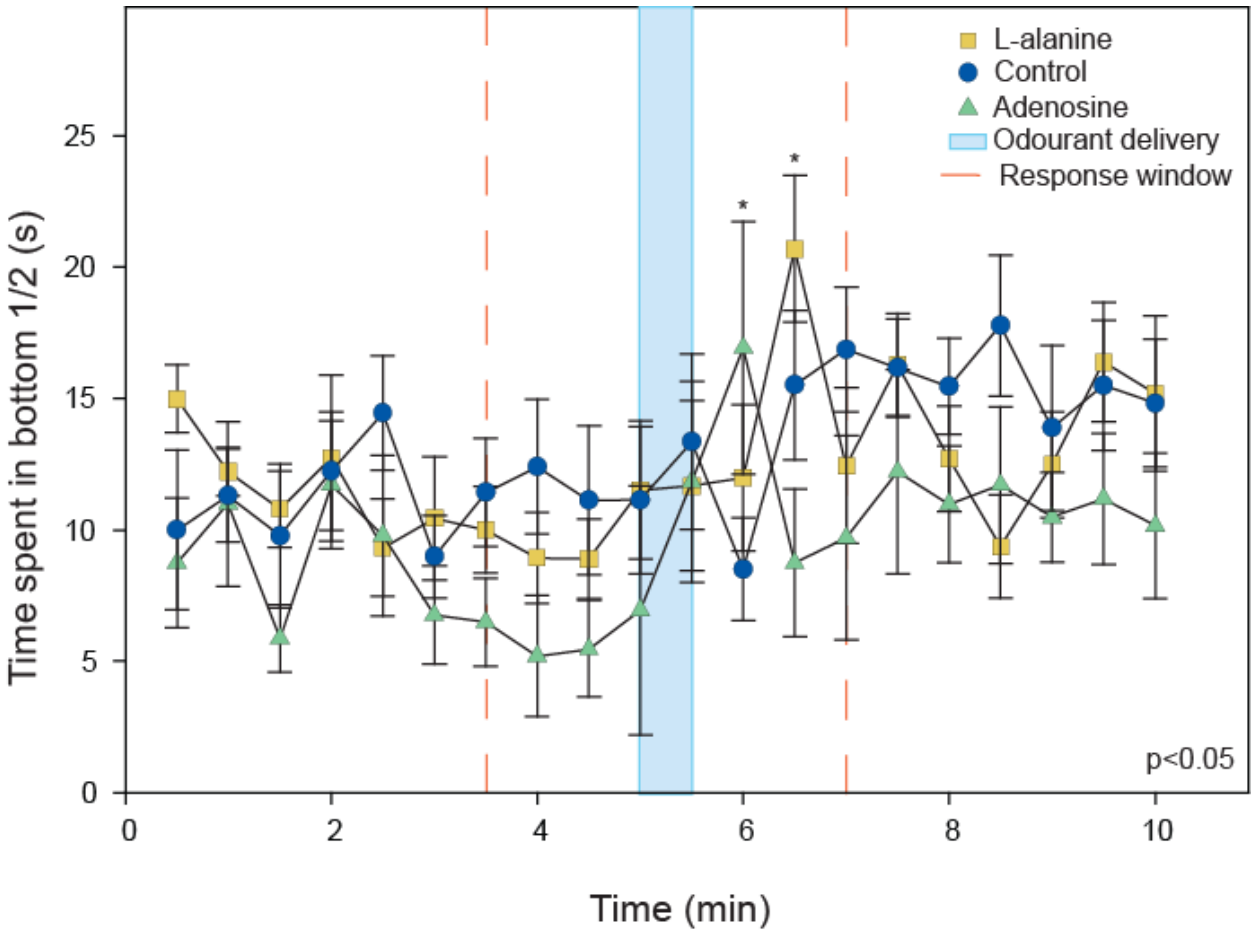


Figure 3.3. Time spent in bottom ½ of tank pre and post odourant exposure. Fish were exposed to $1x10^{-5}$M adenosine, $1x10^{-4}$M L-alanine or fish system water (n=10 per treatment). Time data is presented as average time (mean ± SEM) per 30s bins. Blue segment denotes odourant addition window. Orange dashed lines denote 4 minute window used for comparison of pre-delivery and post-delivery activity. Asterisks denote adenosine minute 6 vs. 5 min significance and L-alanine 6.5 vs. 3.5-6 min.

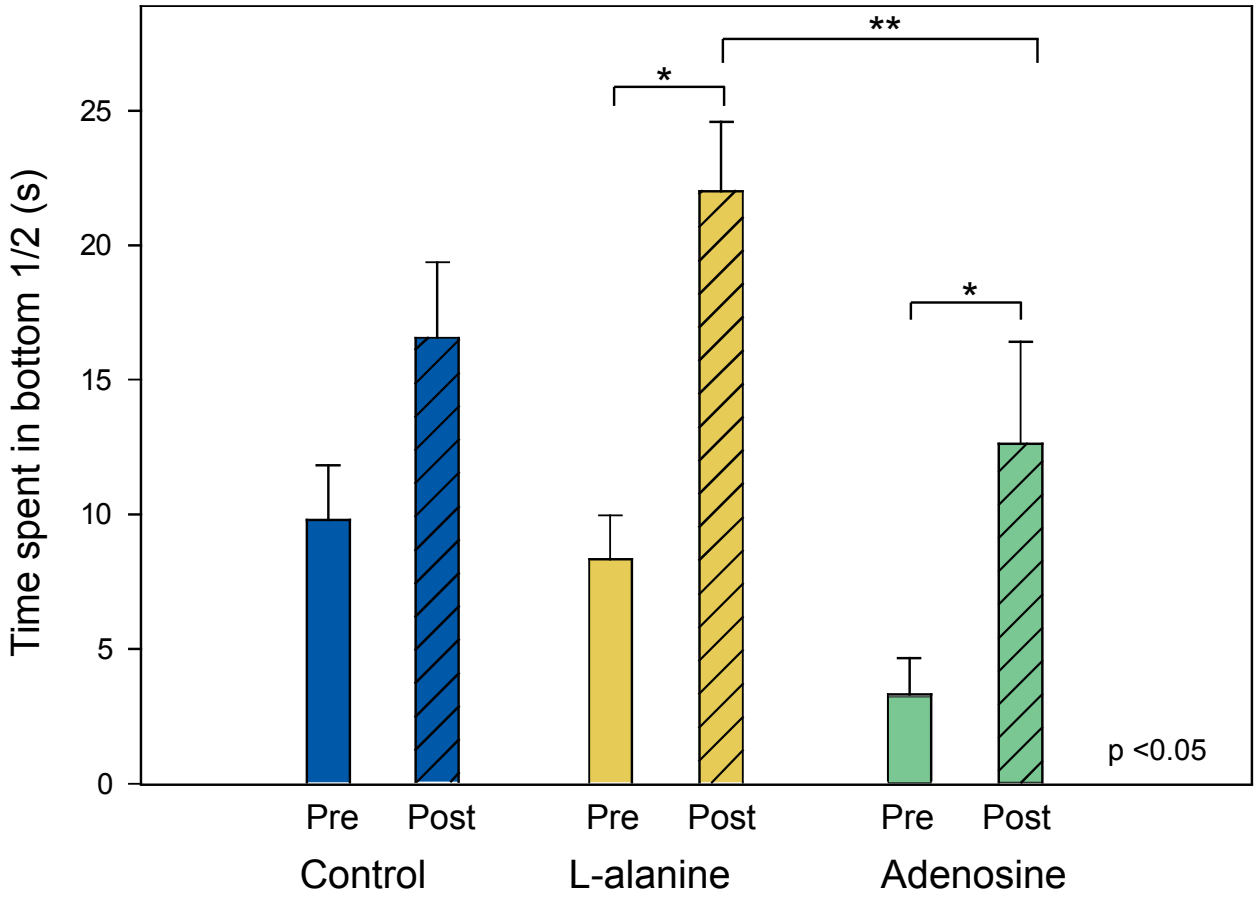


Figure 3.4. Spike alignment for time spent in bottom ½ of tank pre and post odourant exposure. Fish were exposed to $1x10^{-5}$M adenosine, $1x10^{-4}$M L-alanine or fish system water (n=10 per treatment). Pre-exposure data is presented as average time (mean ± SEM) spent in the bottom ½ per 30s bins during the 2 minutes before exposure. Post exposure data is presented as average time spent in the bottom ½ for the maximum % change value for each fish compared to their pre-exposure average. Single asterisk denotes significance between pre and post periods. Double asterisk denotes significance between treatments during the post odourant period.

Larval zebrafish avoidance attraction responses to odourant set 1: Manual scoring vs. videogram results.

Embryo medium

Control trials conducted with EM on both sides of the apparatus resulted in varying distributions across the apparatus (Figure 3.5). Similar to preliminary apparatus validation trials, 5 dpf fish initially displayed increased occupancy in the middle chamber at time points 10, 20, 30, 45, 60, 90 ,120, 180 and 240s having on average 84.1 ± 2.7, 76.9 ± 4.0, 73.2 ± 4.8, 68.4 ± 4.4, 62.5 ± 4.8, 57.5 ± 6.1, 54.3 ± 5.0, 49.4 ± 5.3 and 48.4 ± 6.0% occupancy respectively . Due to this higher occupancy in the middle chamber, the larval distribution significantly differed from the expected 33.3% per chamber (n=10 trials, 9-11 fish per trial; left vs. middle vs. right heterogeneous chi square test: HCS $\chi^2_{0.05,\,18}$ =5.05, 9.06, 11.88, 12.89, 15.14, 21.85, 14.85, 14.45, 16.83, pooled corrected $\chi^2_{0.05,\,2}$ = 112.75, 79.63, 68.59, 52.99, 36.25, 24.54, 19.50, 19.24, 10.32). However, from 300s on, fish occupancy met the expected 1/3:1/3:1/3 distribution with a mean occupancy in the middle chamber of 44.3 ± 5.2, 45.5 ± 4.7, 35.6 ± 5.6% (left vs. middle vs. right heterogeneous chi square test: HCS $\chi^2_{0.05,\,18}$ =11.76, 12.81, 14.92, pooled corrected $\chi^2_{0.05,\,2}$ =4.50, 5.46, 0.62). For 300, 420 and 600s the left and right chambers had a mean occupancy of 27.9 ± 2.9, 26.7± 3.3, 28.7 ± 3.5% and 27.7 ± 3.1, 27.9 ± 3.7, 35.8 ± 3.4% respectively.

Videogram analysis of the same EM exposed 5dpf trials also showed equal area use of the apparatus. The average centre of mass shifts for 5 dpf EM trials differed minimally from the apparatus centre or point 'zero'. : -1.8 ± 2.6, -1.5 ± 1.8, -1.6 ± 1.7, 0.5 ± 1.2, 2.0 ± 1.0 and 1.8 ±1.3mm (n= 9 for time intervals of 4-5 and 7-10 min).

Manually scored 6 dpf control trials also showed an initial weighted proportion of larvae in the middle chamber, but only until 180s (n=10 trials,10-11 fish per trial, left vs. middle vs. right heterogeneous chi square test: HCS $\chi^2_{0.05, 18}$ = 6.90, 7.08, 20.46, 18.13, 17.54, 21.74, pooled corrected $\chi^2_{0.05, 2}$ = 52.30, 45.40, 25.80, 16.41, 11.73, 6.60) Data from 120s violated the heterogeneous chi square test and could not be pooled for analysis (HCS $\chi^2_{0.05, 18}$ =29.46). The initial mean percentage of fish in the middle chamber from 10-120s was 67.5 ± 3.5, 65.4 ± 3.4, 56.6 ± 5.5, 51.7 ± 4.8, 48.5 ± 4.1, 43.6 ± 4.1 and 32.8 ± 6.2%.

At 180s, the distribution across chambers met the expected 33.3% with 39.5 ± 4.9, 27.8 ± 3.9 and 32.6 ± 4.7% occupancy in the left, middle and right chambers respectively (left vs. middle vs. right heterogeneous chi square test: HCS $\chi^2_{0.05, 18}$ =16.5 pooled corrected $\chi^2_{0.05, 2}$ = 1.8). Although the 240 - 420s time points also fit a 1/3:1/3:1/3 distribution, they were found to better fit a 2/5:1/5:2/5 as middle chamber occupancy decreased (left vs. middle vs. right heterogeneous chi square test: HCS $\chi^2_{0.05, 18}$ = 18.78, 27.27, 13.28, pooled corrected $\chi^2_{0.05, 2}$ = 0.35, 0.47, and 2.43). The mean middle chamber percent occupancies of 22.9 ± 3.7, 22.5 ± 5.4 and 19.7 ± 3.6% were lower than those of the left and right chambers (39.5 ± 5.3, 40.7 ± 6.6, 47.5 ± 4.4% and 37.5 ± 5.2, 36.7 ± 4.3, 32.7 ± 3.7% respectively). At 600s, fish were observed to be primarily, but evenly, positioned in the left or right chambers with mean percentages of fish per left, middle and right of 46.5 ±3.9, 10.0 ± 3.0 and 43.5 ± 2.8% respectively. Data at 600s best fit a 4.5/10:1/10:4.5/10 distribution (left vs. middle vs. right heterogeneous chi square test: HCS $\chi^2_{0.05, 18}$ = 12.78, pooled corrected $\chi^2_{0.05, 2}$ =0.06).

6 dpf EM videogram centre of mass data also showed even left-right distribution with mean shift values near zero: -3.6 ± 3.5, -0.3 ± 3.5, 0.8 ± 3.6, -2.0 ± 3.2, 1.9 ± 3.0 and 2.6 ± 2.3mm. (n= 9 for 4-5 min).

Similar to 5 and 6 dpf EM trials, manually scored control trials for 7 dpf larvae also showed an initial skewed occupancy for the middle chamber from 10-180s with mean values of 86.0 ± 2.7, 76.0 ± 3.7, 76.0 ± 4.3, 72.0 ± 5.7, 68.0 ± 6.1, 55.0 ± 7.9, 56.0 ± 5.4 and 44.0 ± 5.0%. (n=10 trials, 10 fish per trial, left vs. middle vs. right heterogeneous chi square test: HCS $\chi^2_{0.05, 18}$ = 5.03, 8.90, 11.97, 21.20, 22.33, 31.55, 17.38, 17.60, pooled corrected $\chi^2_{0.05, 2}$ = 121.76, 79.45, 79.39, 64.98, 52.09, 20.22, 24.73, 6.00). Data from 90s could not be used for analysis due to heterogeneity violation.

Fish showed an even 1/3:1/3:1/3 distribution from 240-420s with an average of 36.0 ± 3.7, 38.0 ± 4.7 and 39.0 ± 4.6% on the left, 34.0 ± 5.0, 32.0 ± 5.7 and 29.0 ± 5.7% in the middle and 30.0 ± 3.9, 30.0 ± 3.9 and 32.0 ± 4.2% on the right (left vs. middle vs. right heterogeneous chi square test: HCS $\chi^2_{0.05, 18}$ = 16.81, 18.980, 19.04, pooled corrected $\chi^2_{0.05, 2}$ = 0.40, 0.78, 1.26). At 600s, larval distribution best fit 2/5:1/5:2/5 with a mean occupancy of 40.0 ± 5.2, 23.0 ± 4.7 and 37.0 ± 4.7% per the left, middle and right chambers respectively (left vs. middle vs. right heterogeneous chi square test: HCS $\chi^2_{0.05, 18}$ = 21.08 pooled corrected $\chi^2_{0.05, 2}$ = 0.48). Overall, control fish moved primarily to the left and right chambers in relatively equal proportion.

The equal use of left and right chambers was supported by the minimal left-right shifts in centre of mass for 7 dpf control trials. As with 5 and 6 dpf control trials, average shift values deviated minimally from zero: -2.1 ± 2.9, -1.1 ± 2.6, -0.9 ± 3.4, 0.7 ± 3.0, 1.4 ± 2.9 and 1.9 ± 3.2mm (n= 9 for time intervals of 3-4 and 4-5 min).

Although fish distributed more to the left and right chambers of the apparatus at 6 and 7dpf manual scoring and videogram analysis showed relatively equal use of the left and right

chambers. Distribution and centre of mass shift in odourant trials could be justifiable compared to EM values.

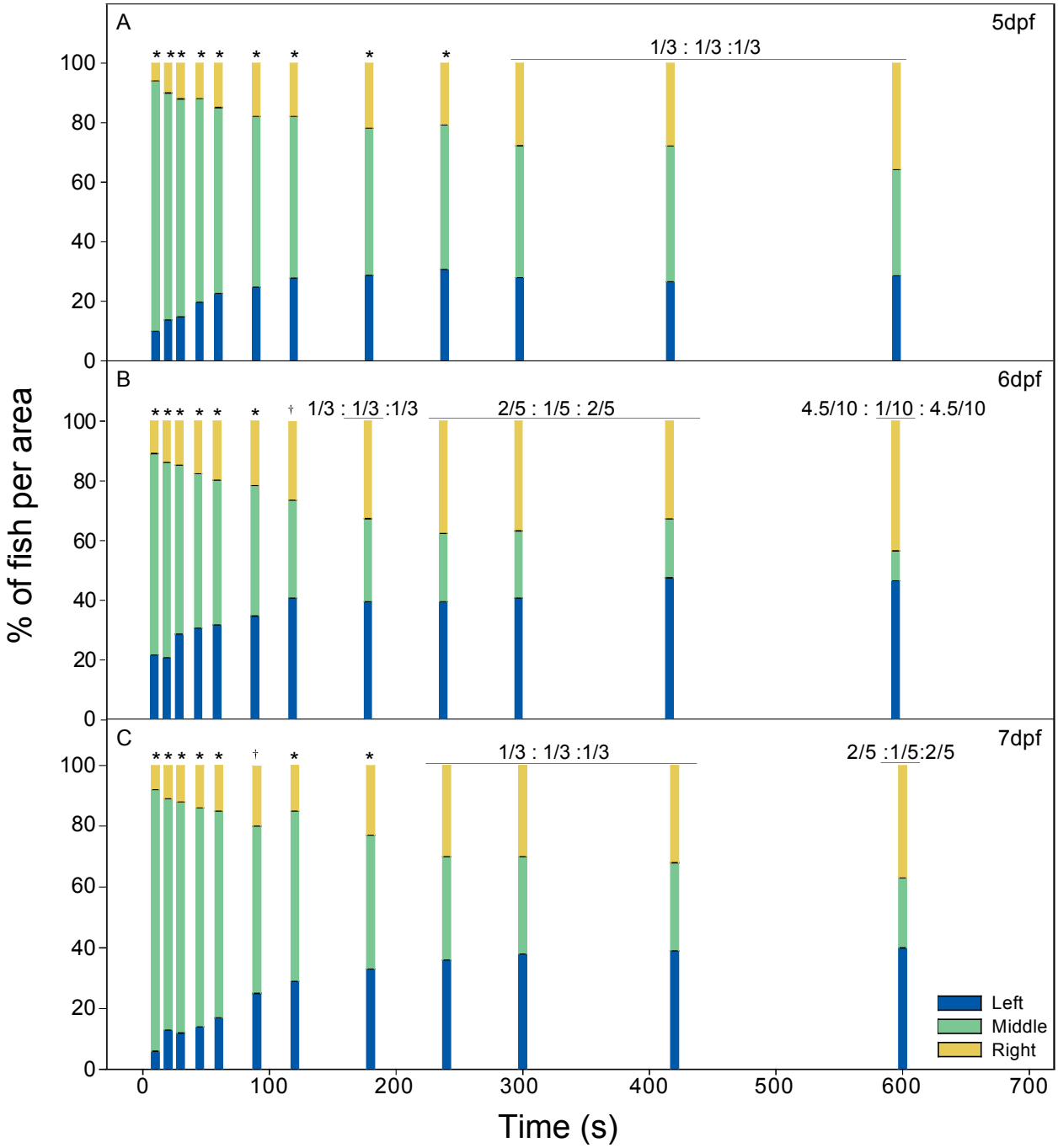


Figure 3.5. Manually scored area occupancy for larvae in control embryo medium (EM) trials. A-C show 5, 6 and 7 dpf trials respectively with n=10 trials per treatment (9-11 fish per trial). Data is presented as the mean percent of fish per area (left, middle or right) at each time point. Goodness of fit determined by heterogeneity chi squared tests. Asterisk denotes rejection of expected even distribution. Dagger identifies data sets that violated heterogeneity. Distributions best fitting the data are listed above each data point.

Adenine

Manual scoring revealed that a greater percentage of 5 dpf larvae occupied the non-odourant area compared to the adenine area (Figure 3.6). At 300 and 420s the mean percent occupancy for the odourant zone was 21.1± 3.9 and 20.0 ± 4.1% compared to the expected 33.3% (n= 9 trials, 10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,8} = 4.87$, 5.37; pooled corrected $\chi^2_{0.05,\,1} = 5.51$, 6.61). At 600s the mean percent occupancy of 25.6 ± 5.0% met the expected ratio (HCS $\chi^2_{0.05,\,8} = 8.18$; pooled corrected $\chi^2_{0.05,\,1} = 2.11$). No variations from the control distributions were observed in either 6 or 7 dpf adenine trials (6 dpf: n= 10 trials, 10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9}$ =7.19, 6.67, 7.04, 7.67, 6.46; pooled corrected $\chi^2_{0.05,\,1} = 0.36$, 3.76, 3.01, 1.26, 0.82, 7 dpf: n= 9 trials, 10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,8} = 7.90$, 5.80, 11.70, 12.50; pooled corrected $\chi^2_{0.05,\,1}$=0.61, 1.01, 0.31, 0.29).

Interestingly, videogram analysis also revealed a centre of mass shift away from the odourant side at 5 dpf, but at a later time interval than observed by manual scoring (Figure 3.7). Although

the overall mean shift values were not affected by the treatment, (two way repeated measures ANOVA*; Ftreatment*$_{1, 109}$=2.264, p =0.151, adenine n= 8 at 1-2 and 3-4 min), in the 7-10 min time interval, the centre of mass shift for adenine was greater than EM (adenine vs. EM: -6.0 ± 3.5mm vs. 1.8 ± 1.3mm; Holm-Sidak p = 0.048). Similar to manual scoring results, adenine treatment did not affect centre of mass shifts for 6 dpf or 7 dpf trials (two way repeated measures ANOVA: 6 dpf *Ftreatment*$_{1, 106}$=0.0762, p =0.786; 7 dpf *Ftreatment*$_{1, 110}$ =0.0115, p =0.916, adenine n= 8 at 3-4 min; Holm-Sidak p>0.05). In summary, both methods of analysis supported an avoidance of the adenine zone at 5dpf.

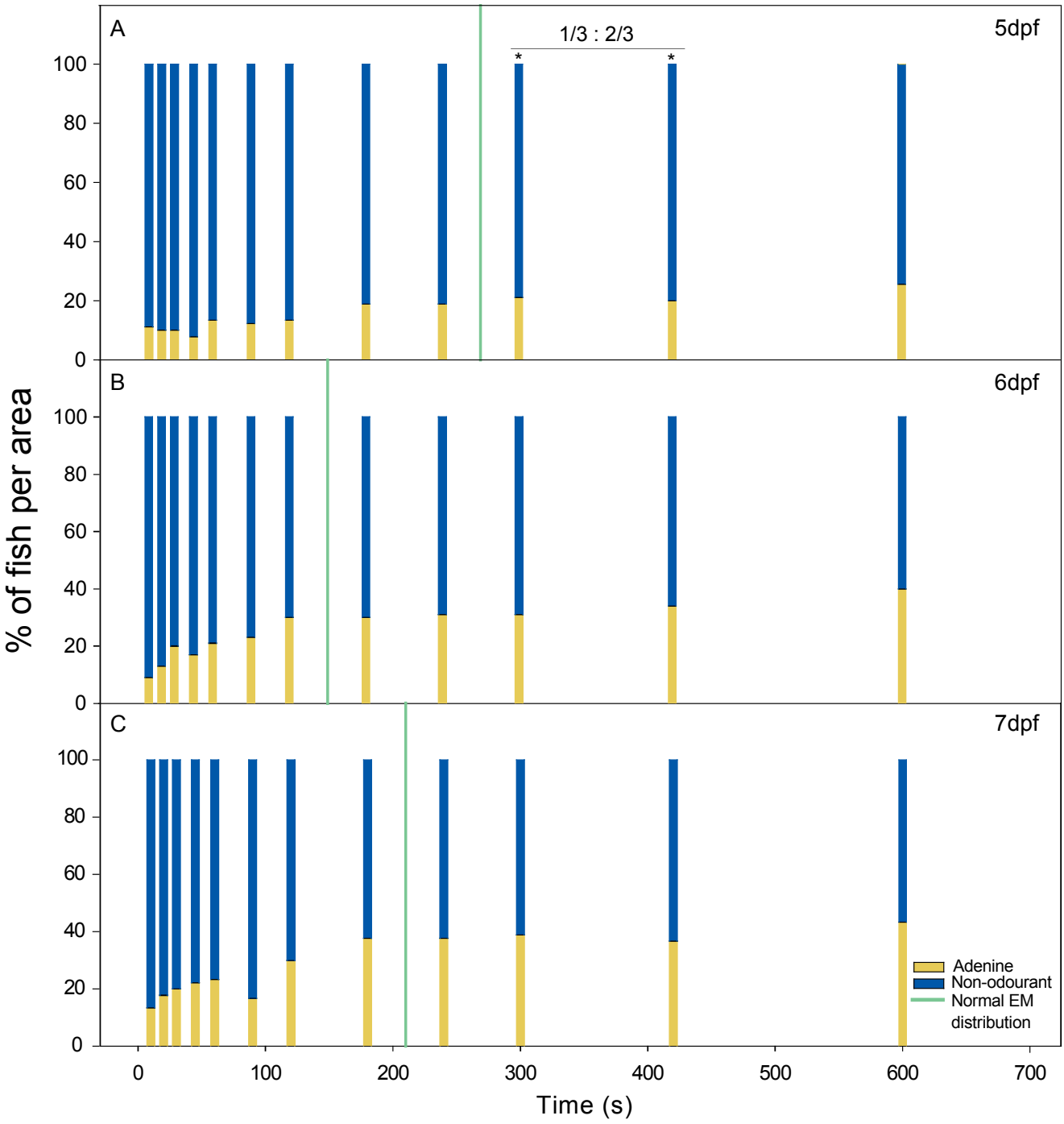

A
5dpf
1/3 : 2/3
*
*
B
6dpf
C
7dpf
% of fish per area
Time (s)
0
100
200
300
400
500
600
700
0
20
40
60
80
100
Adenine
Non-odourant
Normal EM distribution

Figure 3.6. Manually scored area occupancy for larval $1x10^{-5}$M adenine trials. A-C show 5, 6 and 7 dpf trials respectively (n=9 trials for 5 and 7 dpf and n=10 trials for 6 dpf trials, n= 10 fish per trial). Data is presented as mean percent of fish per area (odourant vs. non-odourant). The green line represents the division between even and skewed EM distributions. Only time points past the green line were analyzed for goodness of fit using heterogeneity chi squared testing. Asterisks denote differences from the expected EM determined 1/3:2/3 odourant: non-odourant distribution.

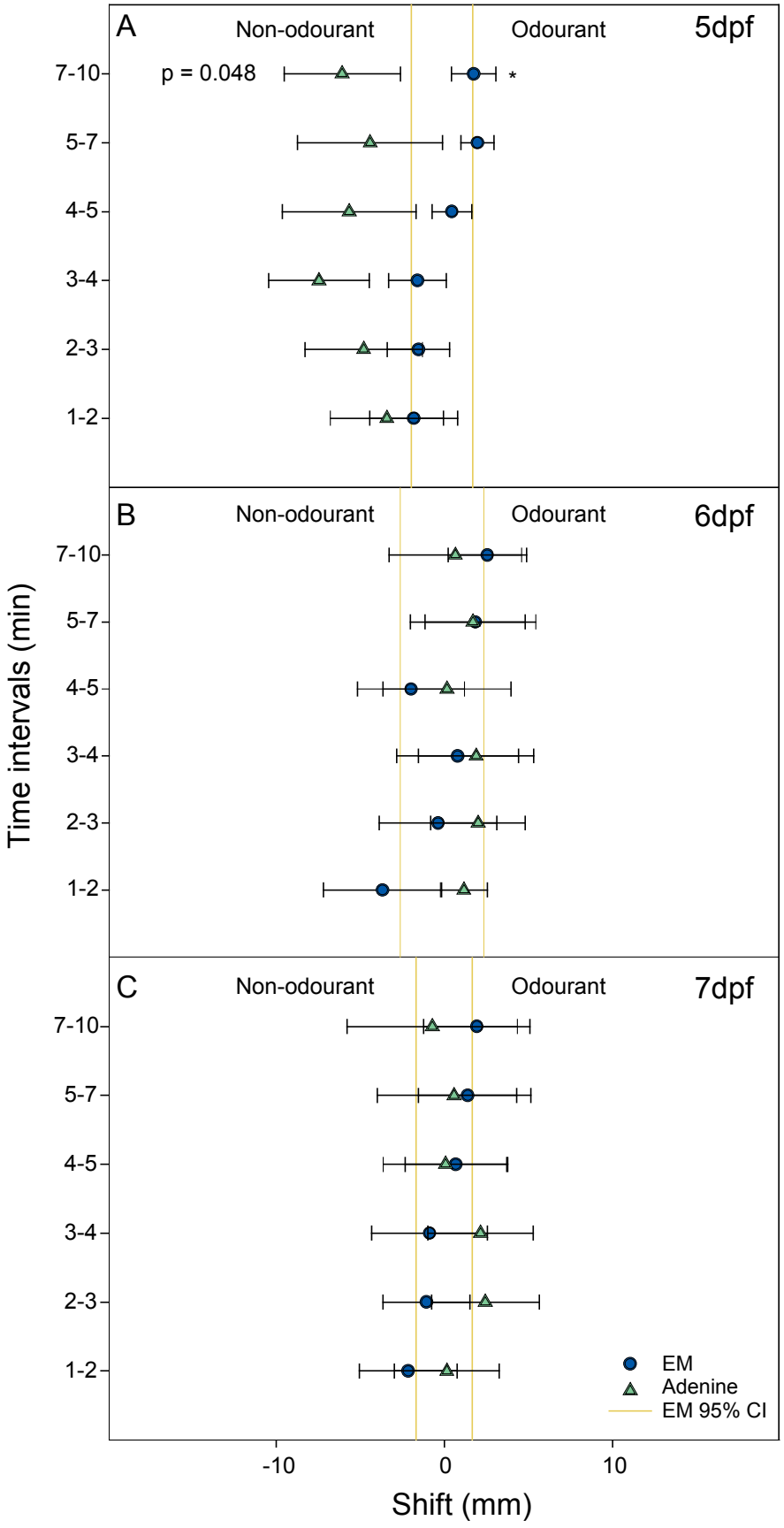

A
Non-odourant
Odourant
5dpf
p = 0.048
*
7-10
5-7
4-5
3-4
2-3
1-2
B
Non-odourant
Odourant
6dpf
C
Non-odourant
Odourant
7dpf
Time intervals (min)
EM
Adenine
EM 95% CI
-10
0
10
Shift (mm)

Figure 3.7. Centre of mass shift (mm) in $1x10^{-5}$M adenine vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10 and n=9 at 4-5 and 7-10min. Adenine n=9 n=8 at 1-2 and 3-4 min. B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. Adenine n=9. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. Adenine n=9 and n=8 at 3-4 min. A –C) Data presented as mean shift in centre of mass± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively. Asterisk denotes significant difference from EM shift.

Adenosine

Fish percent occupancy in 5dpf adenosine trials did not differ from control distributions when trials were manually scored (Figure 3.8; n= 10 trials, 10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9}$ = 10.06, 8.26, 8.29; pooled corrected $\chi^2_{0.05,\,1}$ = 3.51, 3.51, 0.21). Distribution was also no different from the control in 6 dpf trials from until 420s. However at 600s, the mean percent occupancy in the adenosine zone (33.1 ± 6.9%) was lower than control (45.0%) indicating odourant avoidance. (n= 10 trials, 9-10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9}$ = 9.02, 9.67, 12.95, 11.25, 14.99; pooled corrected $\chi^2_{0.05,\,1}$ = 0.56, 0.40, 3.49, 1.57, 4.98). No deviations from the control distributions were observed in 7 dpf adenosine trials (n= 10 trials, 10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9}$ = 8.51, 3.78, 10.85, 8.71; pooled corrected $\chi^2_{0.05,\,1}$ = 0.66, 2.10, 0.00, 0.01). In summary, manual scoring showed adenosine avoidance only at 6 dpf.

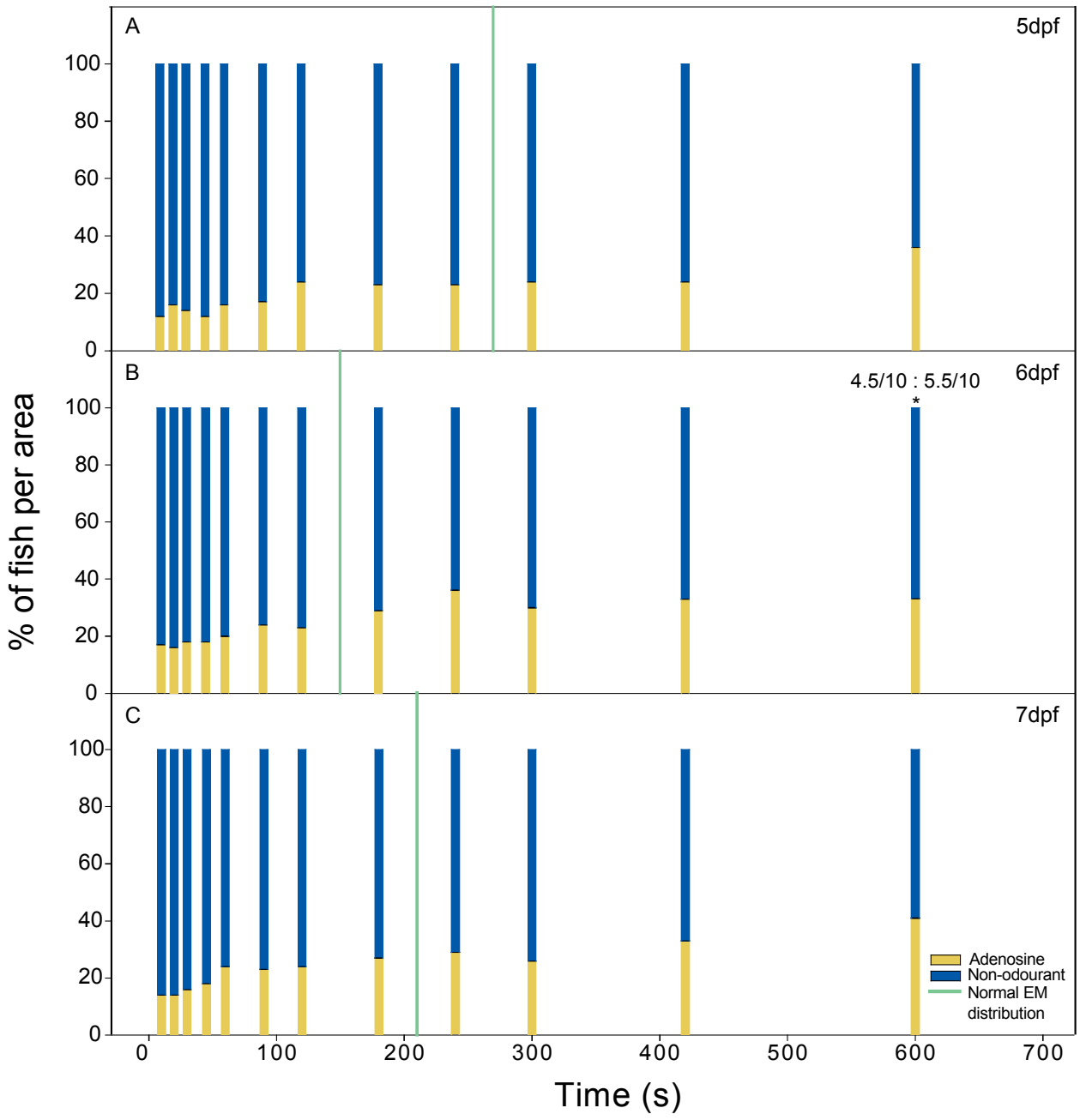

A
5dpf
B
6dpf
4.5/10 : 5.5/10
*
C
7dpf
% of fish per area
Time (s)
Adenosine
Non-odourant
Normal EM distribution

Figure 3.8. Manually scored area occupancy for larval $1x10^{-5}$M adenosine trials. A-C show 5, 6 and 7 dpf trials respectively (n=10 trials per age and n=9-10 fish per trial). Data is presented as mean percent of fish per area (odourant vs. non-odourant). The green line represents the division between even and skewed EM distributions. Only time points past the green line were analyzed for goodness of fit using heterogeneity chi squared testing. Asterisk denotes difference from the expected EM determined 4.5/10: 5.5/10 odourant: non-odourant distribution.

Videogram analysis of adenosine trials showed no effect of treatment on centre of mass shift for 5, 6 or 7 dpf (Figure 3.9; two way repeated measures ANOVA: 5 dpf $Ftreatment_{1, 117}$=0.830, p =0.374; 6 dpf $Ftreatment_{1, 109}$ =0.0231, p =0.881, adenosine n=9 at 3-4 min and n=8 at 4-5 min; 7 dpf $Ftreatment_{1, 117}$ =0.111, p =0.742). However at 5 dpf, the effect of the treatment increased with time ($F_{1, 117}$=4.872, p<0.001). This was reflected by a notable centre of mass shift away from the adenosine zone at the 7-10 min interval (adenosine vs. EM: -5.2 ± 2.7mm vs. 1.8 ± 1.3mm; Holm-Sidak p = 0.056). The avoidance of adenosine at 6 dpf determined by manual scoring was not observed in the centre of mass data, but was instead seen at 5 dpf similar to adenine results.

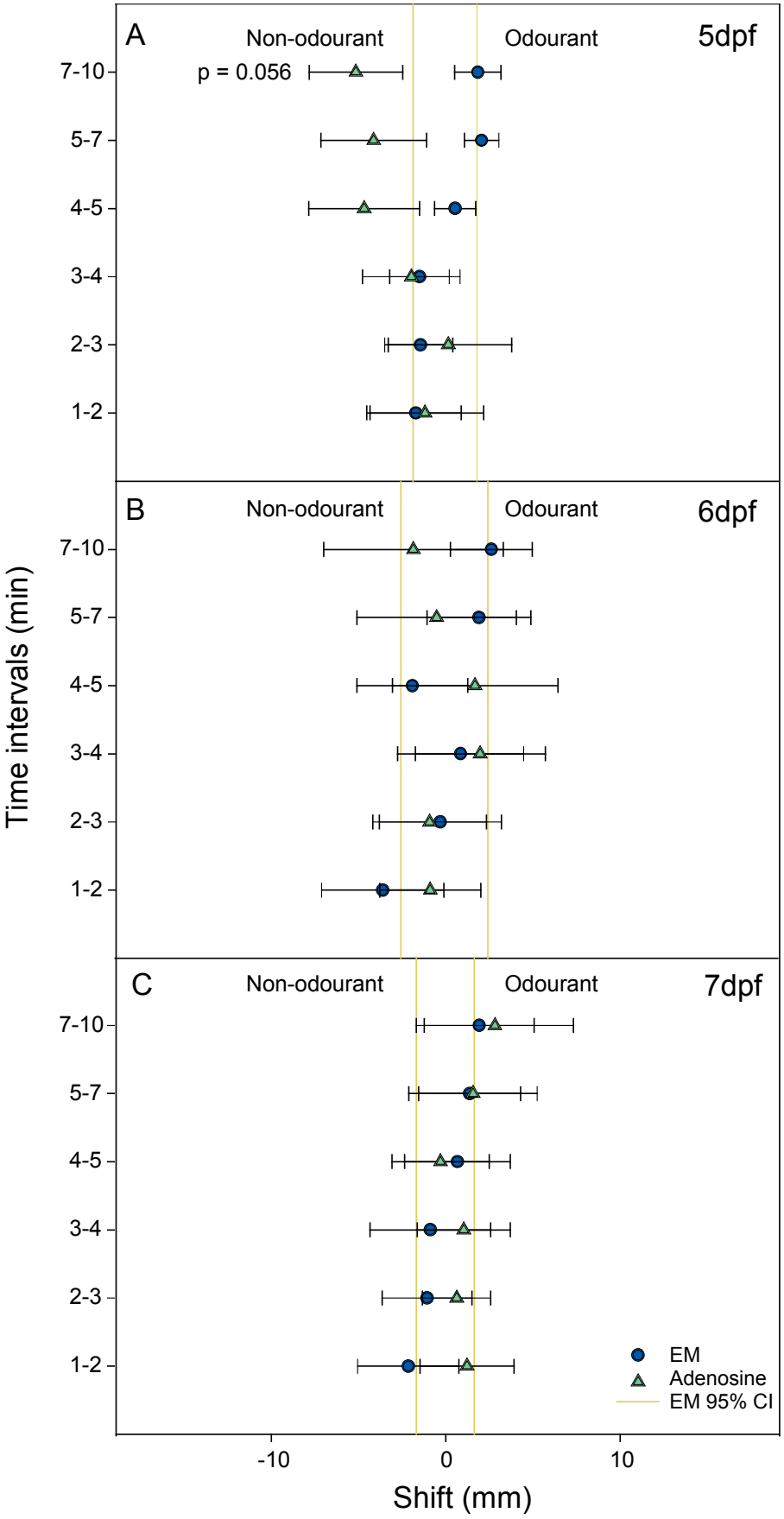

A
Non-odourant
Odourant
5dpf
p = 0.056
B
Non-odourant
Odourant
6dpf
C
Non-odourant
Odourant
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
Time intervals (min)
-10
0
10
Shift (mm)
EM
Adenosine
EM 95% CI

Figure 3.9. Centre of mass shift (mm) in $1x10^{-5}$M adenosine vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10 and n=9 at 4-5 and 7-10 min. Adenosine n=10. B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. Adenosine n=10 and n=9 at 3-4 min and n=8 at 4-5 min. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. Adenosine n=10. A –C) Data presented as mean shift in centre of mass ± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively.

Adenosine-5'-monophosphate

Larvae aged 5 dpf exhibited no change in chamber distribution compared to control trials when AMP was present (Figure 3.10; AMP n= 10 trials, 9-10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9}$ = 8.47, 14.08, 10.78; pooled corrected $\chi^2_{0.05,\,1}$=0.03, 0.00, 3.09). At 6 dpf however, larvae exhibited a transient increased occupancy in the non-odourant zone compared to EM distributions (71.0 ± 5.3% and 67.0 ± 7.1% vs. the expected 60.0% and 55.0% at 300 and 600s respectively; AMP n= 10 trials, 10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9}$ =10.30, 9.21, 8.71, 7.67, 15.39; pooled corrected $\chi^2_{0.05,\,1}$=0.66, 1.76, 4.59, 1.26, 5.34). Fish tested at 7 dpf also showed increased occupancy in the non-odourant zone (78.4 ± 2.0% at 240s and 77.3 ± 2.7% at 300s compared to the expected 66.7%;AMP n= 9 trials, 9-10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,8}$ = 1.29, 2.45, 2.08, 1.50; pooled corrected $\chi^2_{0.05,\,1}$= 4.94, 3.99, 0.41, 2.81). Post 300s, larval percent occupancy met expected control distributions. Overall, manual scoring showed transient avoidance of AMP areas at 6 and 7 dpf.

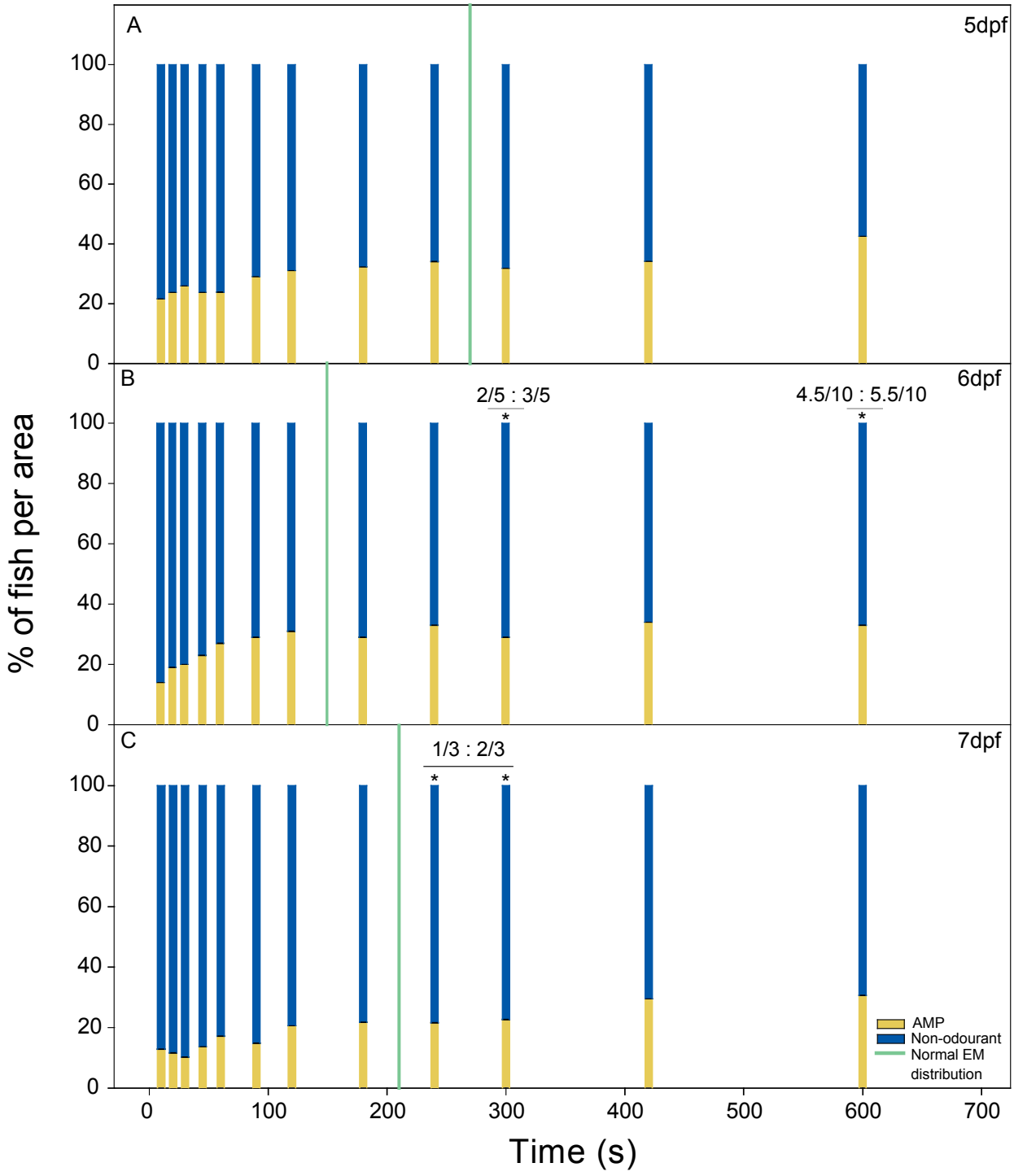

A
5dpf
B
6dpf
C
7dpf
2/5 : 3/5
4.5/10 : 5.5/10
1/3 : 2/3
*
% of fish per area
Time (s)
0
20
40
60
80
100
0
100
200
300
400
500
600
700
AMP
Non-odourant
Normal EM distribution

Figure 3.10. Manually scored area occupancy for larval $1x10^{-5}$M AMP trials. A-C show 5, 6 and 7 dpf trials respectively (n=10 trials for 5 and 6 dpf, n=9 trials for 7 dpf, and n=9-10 fish per trial). Data is presented as mean percent of fish per area (odourant vs. non-odourant). The green line represents the division between even and skewed EM distributions. Only time points past the green line were analyzed for goodness of fit using heterogeneity chi squared testing. Asterisks denote differences from the expected EM determined odourant: non-odourant distributions. Expected distributions are noted above asterisks.

Centre of mass data for 5 dpf AMP trials also revealed no effect of odourant presence on area use (Figure 3.11; two way repeated measures ANOVA, $Ftreatment_{1, 115}$=0.943, p =0.344, n=8 for 1-2 min). At 6 dpf, although manually scoring the data revealed an avoidance response, odourant presence did not affect centre of mass shift (two way repeated measures ANOVA, $Ftreatment_{1, 104}$=0.00118, p =0.973, AMP n=9 for 1-2 and 5-7 min and n=7 for 2-3 and 3-4 min). There was an insignificant trend for odourant attraction observed at 1-2 min, but effects decreased with increasing time ($Ftreatment\ x\ time_{5, 104}$=4.607, p =0.001). In 7 dpf trials, overall odourant treatment had a subtle effect on centre of mass shift, and although there was a visible trend of increased avoidance, the effect of the odourant did not depend on time (two way repeated measures ANOVA, $Ftreatment_{1, 109}$=3.223, p =0.090, $Ftreatment\ x\ time_{5, 109}$=1.372, p =0.244 AMP n=8 for 1-2 and 7-10 min). Despite minimal overall effects of AMP, there were notable shifts away from the odourant zone at 5-7 min (-6.3 ± 2.5 vs. 1.4 ± 2.9mm; p = 0.069) and 7-10 min (-10.3 ± 3.8 vs. 1.9 ± 3.2mm; (p=0.013). Interestingly, the robust avoidance response was later than that identified by manual scoring. Nevertheless, phosphate addition to adenosine (AMP) still evoked an avoidance response, but at later age(s) than 5dpf.

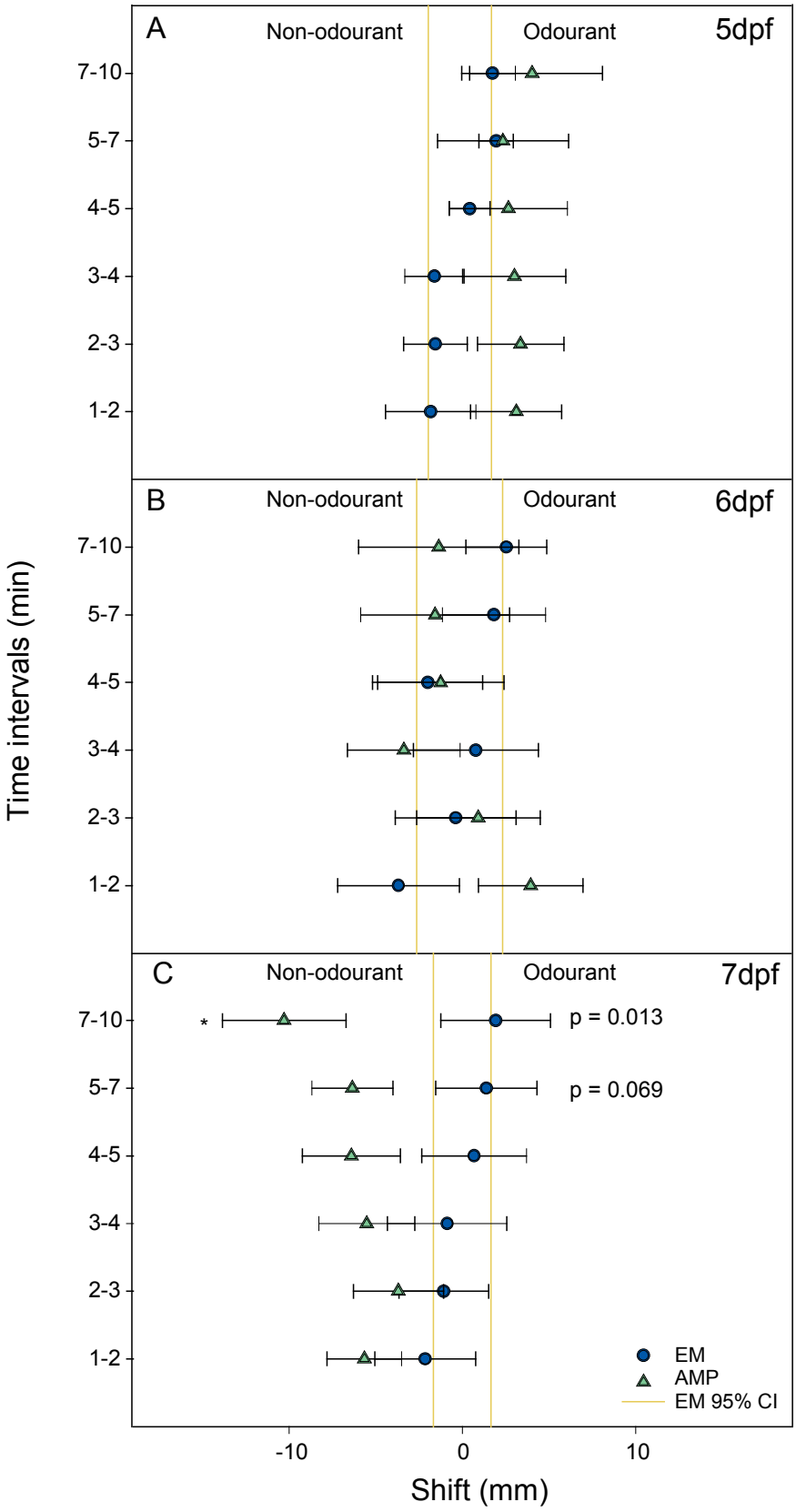

A
Non-odourant
Odourant
5dpf
B
Non-odourant
Odourant
6dpf
C
Non-odourant
Odourant
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
Time intervals (min)
*
p = 0.013
p = 0.069
EM
AMP
EM 95% CI
-10
0
10
Shift (mm)

Figure 3.11. Centre of mass shift (mm) in $1x10^{-5}$M AMP vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10 and n=9 at 4-5 and 7-10 min. AMP n=10, n=8 1-2 min. B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. AMP n=10 and n=9 for 1-2 and 5-7 min and n=7 for 2-3 and 3-4 min. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. AMP n=9, n=8 at 1-2 and 7-10 min. A –C) Data presented as mean shift in centre of mass ± SEM per time intervals (minutes). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively. Asterisk denotes significant difference from EM shift.

Adenosine-5'-triphosphate

Manual scoring of 5 dpf ATP trials showed the same distribution of fish as in control trials (Figure 3.12; ATP n= 9 trials, 9-11 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,8}$=3.38, 3.47, 5.02; pooled corrected $\chi^2_{0.05,\,1}$=1.51, 0.01, 0.01). A reduced percent occupancy in the ATP zone was observed in 6 dpf trials when compared to EM at 180s (19.4 ± 5.0% were in the vs. the expected 33.3%) and at 240 and 300s (, 27.4 ± 5.6% and 26.4 ± 5.4% compared to the expected 40.0%;ATP n= 10 trials, 9-10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9}$=8.20, 9.64, 8.96, 10.85; pooled corrected $\chi^2_{0.05,\,1}$=8.28, 6.16, 7.22, 0.71). Data at 600s could not be analyzed due to failed heterogeneity (HCS $\chi^2_{0.05,\,9}$=18.28). 7 dpf also exhibited avoidance of the ATP zone, but intermittently during the trial period. At 300s, 23.0 ± 5.3% preferred the ATP zone compared to the expected 33.3% and at 600s, 29.1 ± 3.0% preferred ATP vs. the expected 40.0% (ATP n= 10 trials, 9-10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9}$=6.06, 8.80, 8.04, 2.57; pooled corrected $\chi^2_{0.05,\,1}$=1.38, 4.10, 2.56, 4.29).

While manual scoring yielded no difference from control for 5 dpf ATP trials, videogram analysis showed a centre of mass shift towards the odourant side at 1-2 min (Figure 3.13; ATP vs EM: 3.4 ± 1.5 vs. -1.8 ± 2.6mm, two way repeated measures, Holm-Sidak p= 0.062) and 2-3 (ATP vs EM: and 3.5 ± 1.2 vs. -1.5± 1.8mm, two way repeated measures, Holm-Sidak p= 0.069). Overall, odourant treatment did not affect centre of mass shift and the early trend in attraction diminished as the trial progressed (two way repeated measures ANOVA: $Ftreatment_{1, 111}$=0.0496, p =0.826; $Ftreatment\ x\ time_{5, 111}$=5.169, p <0.001). At 6 dpf, there was no difference in the centre of mass shift between ATP and EM treatments (two way repeated measures ANOVA, $Ftreatment_{1, 108}$=0.738, p =0.402; Holm-Sidak p>0.05, ATP n= 8 2-3 min and n=9 for 3-4 and 4-5 min). However, the avoidance response observed by manual scoring was also identified in videogram analysis of 7 dpf trials. A two way repeated measures ANOVA showed no effect of odourant on centre of mass shift, however the odourant's effect on centre of mass did increase with time ($Ftreatment_{1, 116}$=4.200, p =0.055; $Ftreatment\ x\ time_{5, 111}$=2.392, p =0.044, n=8 at 4-5 min, rank transformed). Specifically, the centre of mass shifted away from the ATP area at 5-7 min (ATP vs EM: -3.6 ± 5.5 vs. 1.4 ± 2.9mm, p=0.019) and 7-10 (ATP vs EM: -9.1 ± 2.2 vs. 1.9 ±3.2, Holm-Sidak p= 0.004). Ultimately, the increased number of phosphates from 1 to 3 (AMP to ATP) did not alter the later stage avoidance responses, but did result in the recurrence of behavioural activity seen at 5 dpf in response to the structurally similar nucleobase and nucleoside (adenine and adenosine). However at 5 dpf, the phosphate lacking compounds evoked avoidance, while ATP evoked attraction.

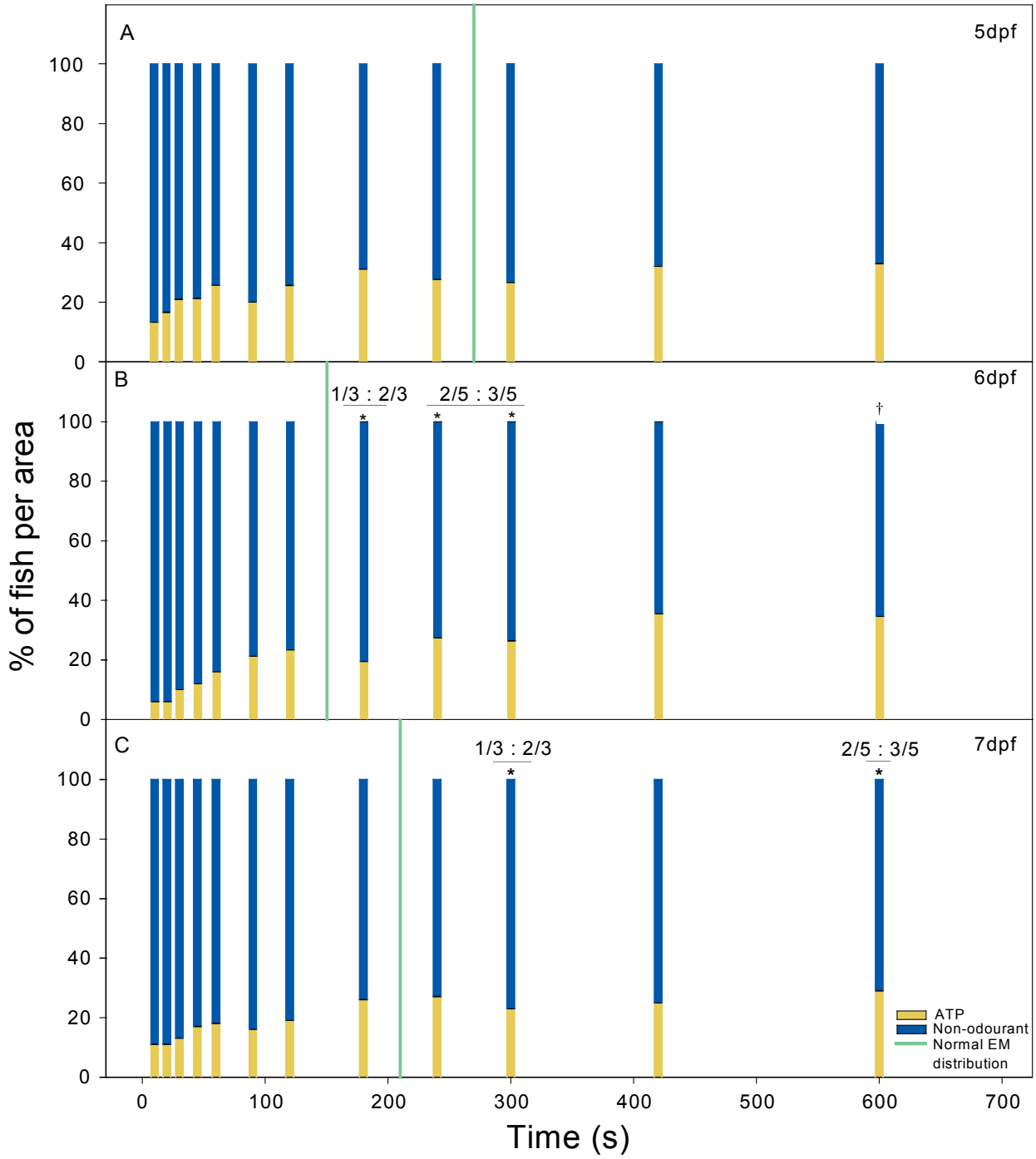

A
5dpf
B
6dpf
C
7dpf
1/3 : 2/3
2/5 : 3/5
1/3 : 2/3
2/5 : 3/5
*
†
% of fish per area
Time (s)
0
20
40
60
80
100
0
100
200
300
400
500
600
700
ATP
Non-odourant
Normal EM distribution

Figure 3.12. Manually scored area occupancy for larval $1x10^{-5}$M ATP trials. A-C show 5, 6 and 7 dpf trials respectively (n=9 trials for 5 dpf and n=10 trials for 6 and 7 dpf; n=9-11 fish per trial). Data is presented as mean percent of fish per area (odourant vs. non-odourant). The green line represents the division between even and skewed EM distributions. Only time points past the green line were analyzed for goodness of fit using heterogeneity chi squared testing. Asterisks denote differences from the expected EM determined odourant: non-odourant distributions. Expected distributions are noted above asterisks. Dagger indicates data that violated heterogeneity testing.

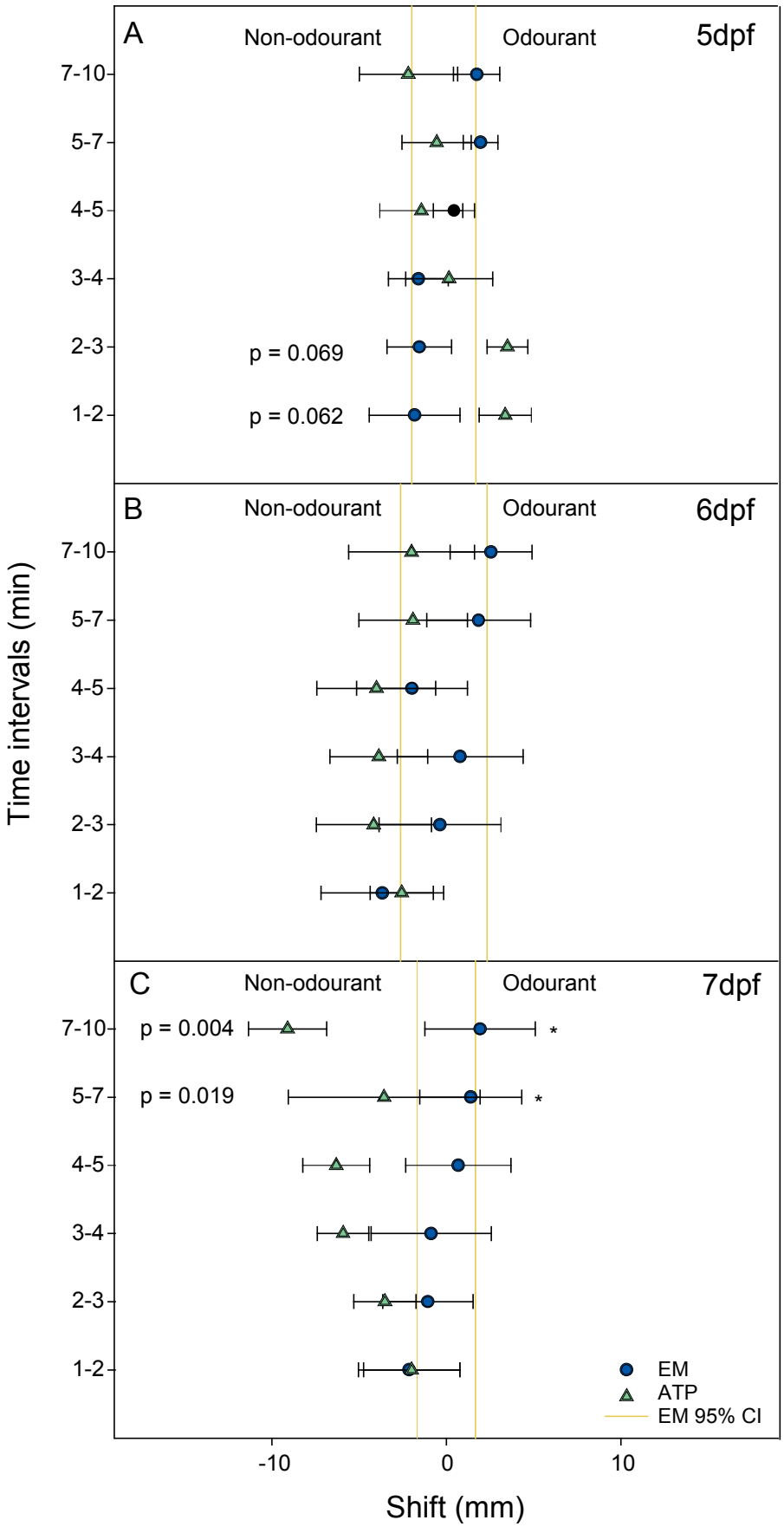

A
Non-odourant
Odourant
5dpf
7-10
5-7
4-5
3-4
2-3
1-2
p = 0.069
p = 0.062
B
Non-odourant
Odourant
6dpf
C
Non-odourant
Odourant
7dpf
p = 0.004
p = 0.019
*
*
EM
ATP
EM 95% CI
Time intervals (min)
-10
0
10
Shift (mm)

Figure 3.13. Centre of mass shift (mm) in $1x10^{-5}$M ATP vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10 and n=9 at 4-5 and 7-10 min. ATP n=9.B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. ATP n=10 n= 8 2-3 min and n=9 for 3-4 and 4-5 min. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. ATP n=10, n=8 at 4-5 min. A –C) Data presented as mean shift in centre of mass ± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively. Asterisks denote significant differences from EM shift.

Guanosine

In response to guanosine, which differs structurally from behaviourally active adenosine by two functional groups on the purine ring, 5 dpf fish trials had no change in distribution compared to EM trials when scored manually (Figure 3.14; guanosine n= 10 trials, 10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9} = 7.23, 5.80, 11.89$; pooled corrected $\chi^2_{0.05,\,1} = 1.53, 0.66, 0.00$). Additionally, at 6 dpf no differences between EM and guanosine were apparent (guanosine n= 10 trials, 10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9} = 7.23, 7.50, 15.04, 11.04, 8.89$; pooled corrected $\chi^2_{0.05,\,1} = 1.53, 3.76, 0.26, 0.84, 0.82$). At 7 dpf also, guanosine did not affect the expected distributions (guanosine n= 9 trials, 9-10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,8} = 7.24, 10.96, 6.79, 12.42$; pooled corrected $\chi^2_{0.05,\,1} = 0.24, 0.07, 0.41, 0.00$).

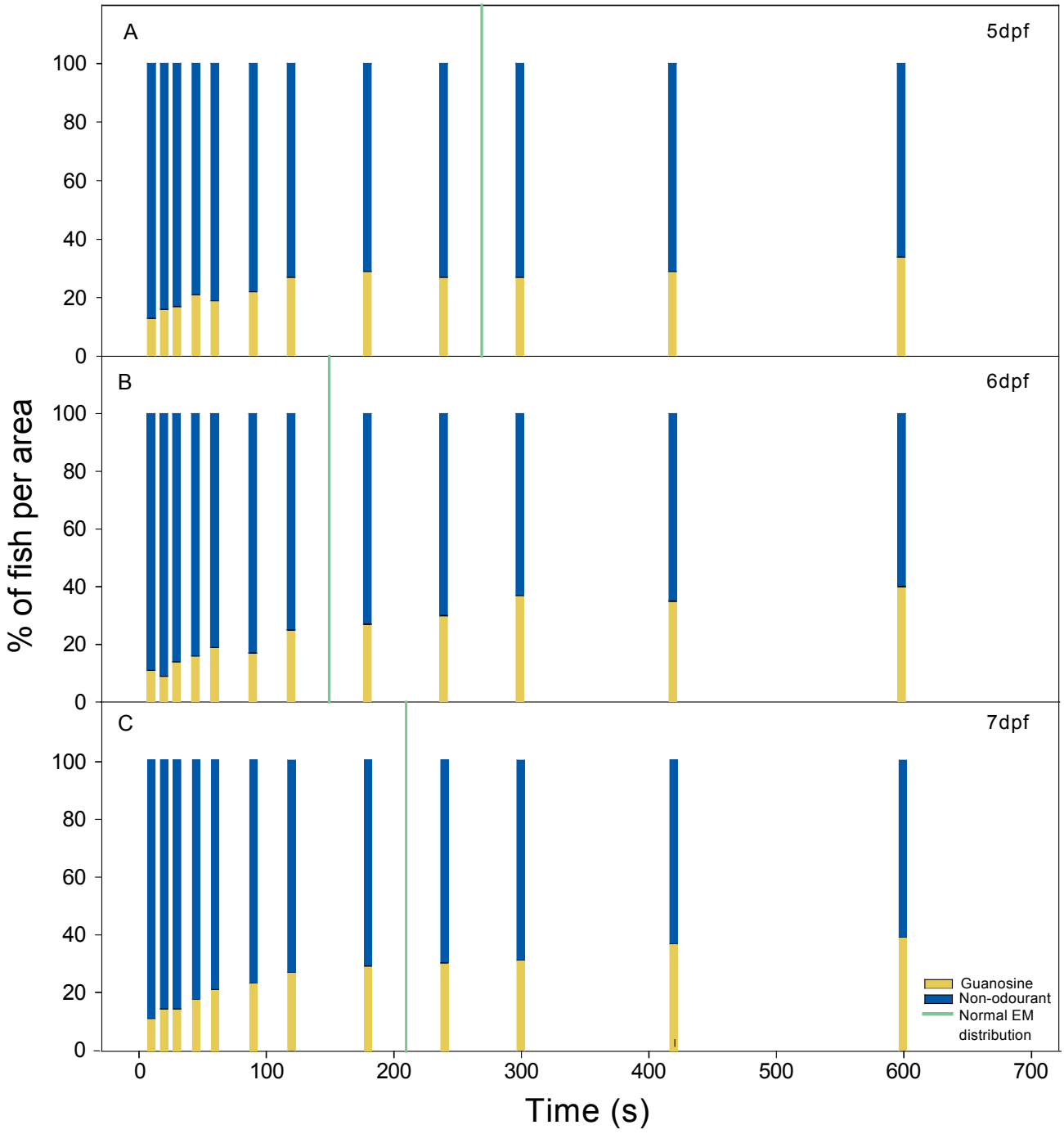

A
5dpf
B
6dpf
C
7dpf
% of fish per area
Time (s)
Guanosine
Non-odourant
Normal EM distribution

Figure 3.14. Manually scored area occupancy for larval $1x10^{-5}$M guanosine trials. A-C show 5, 6 and 7 dpf trials respectively (n=10 trials for 5 and 6 dpf and n=9 trials for 7 dpf; n=9-10 fish per trial). Data is presented as mean percent of fish per area (odourant vs. non-odourant). The green line represents the division between even and skewed EM distributions. Only time points past the green line were analyzed for goodness of fit using heterogeneity chi squared testing.

Similar to manual scoring, centre of mass shifts in response to guanosine presence did not differ from EM trials at 5,6 or 7 dpf (Figure 3.15; two way repeated measures ANOVA: 5 dpf $Ftreatment_{1, 114}$=0.0563, p =0.815, guanosine n=8 2-3 min and n=9 3-4 min; 6 dpf $Ftreatment_{1, 105}$ =0.0274, p =0.871, n= 8 at 1-2 min, rank transformed; 7 dpf $Ftreatment_{1, 110}$ =0.511, p =0.485, n= 8 at 4-5 min, Holm-Sidak p>0.05 for all ages). Both methods of analysis suggested that guanosine; a nucleoside containing the purine ring guanine was neither aversive nor attractive.

Guanosine-5'-monophosphate

The addition of a phosphate to guanosine, making the structure GMP, rendered the compound capable of inducing changes in occupancy behaviour at distinct ages. Manually scored 5 dpf GMP trials revealed no difference in area occupancy between odourant and control trials (Figure 3.16; GMP n= 9 trials, 10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05, 8}$ = 6.38, 4.49, 6.38; pooled corrected $\chi^2_{0.05, 1}$= 2.11, 1.51, 2.81). At 6 dpf however, larvae exhibited avoidance of the GMP area at 180s (21.5 ± 4.6% vs. the expected 33.3%) and both 240 and 300s (25.5 ± 2.2 and 26.5 ± 4.3% respectively vs. the expected 40.0%; GMP n= 10 trials, 10-11 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05, 9}$ = 7.30, 1.63, 5.83, 9.10, 14.06; pooled corrected $\chi^2_{0.05, 1}$= 5.83, 8.35, 7.23, 0.76,

1.62). Fish distribution after 300s did not differ from the observed EM ratios. Larvae did not respond to GMP at 7 dpf as area distributions were not different from those under control conditions (GMP n= 10 trials, 9-10 fish per trial, odourant vs. non-odourant zone heterogeneous chi square test: HCS $\chi^2_{0.05,\,9} = 11.02, 8.55, 8.62, 7.15$; pooled corrected $\chi^2_{0.05,\,1} = 0.56, 0.01, 0.56, 0.19$).

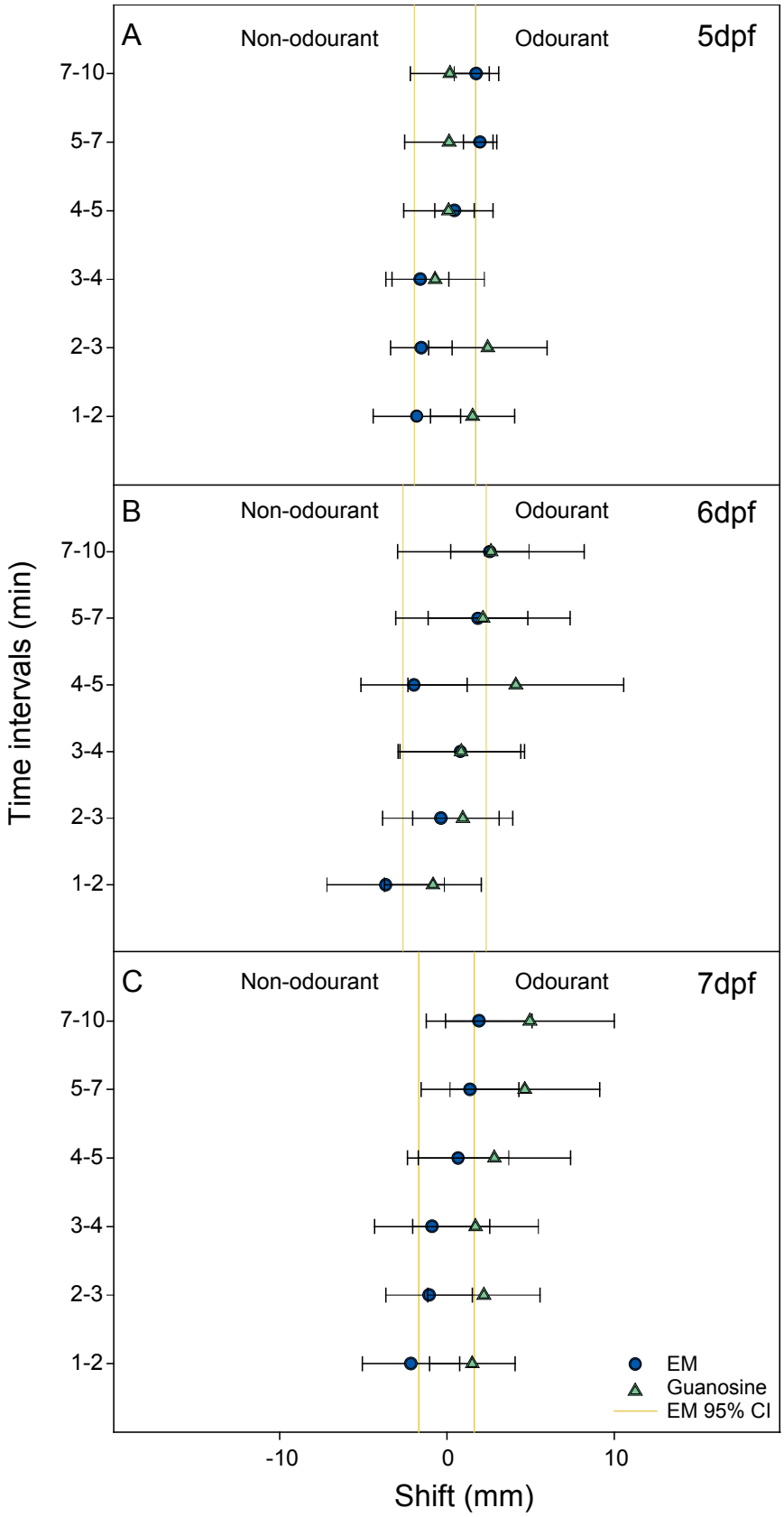

A
Non-odourant
Odourant
5dpf
B
Non-odourant
Odourant
6dpf
C
Non-odourant
Odourant
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
Time intervals (min)
-10
0
10
Shift (mm)
EM
Guanosine
EM 95% CI

Figure 3.15. Centre of mass shift (mm) in 1x 10^{-5}M guanosine vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10 and n=9 at 4-5 and 7-10 min. Guanosine n=10 and at 2-3 and 3-4 min, n=8 and n=9 respectively B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. Guanosine n=10 and n= 8 at 1-2 min. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. Guanosine n=9 and n=8 at 4-5 min. A –C) Data presented as mean shift in Centre of mass ± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively.

Videogram analysis compared to manual scoring yielded different response types and response ages for GMP. Overall, a two way repeated measures ANOVA showed that at 5 dpf GMP treatment did not affect centre of mass shift, but the effect of the odourant treatment did depend on the time bin (Figure 3.17; $Ftreatment_{1, 110}$=1.164, p =0.296; $Ftreatment\ x\ time_{5, 110}$=3.336, p =0.009, GMP n=8 at 1-2 min, rank transformed). There was a prominent shift towards the GMP side at both 2-3 min (GMP vs EM: 3.5 ± 1.9 vs. -1.5 ± 1.8mm, Holm-Sidak p= 0.085) and 3-4 min (GMP vs EM: 5.4 ± 2.9 vs. -1.6 ± 1.7mm, Holm-Sidak p= 0.029). This shift towards the odourant zone was indicative of attraction, a response not seen in manually scored data. Interestingly, the 6 dpf avoidance of GMP observed by manual scoring was mirrored by a negative centre of mass shift trend, but the data was not significantly different from EM values (two way repeated measure ANOVA: $Ftreatment_{1, 111}$=0.182, p =0.675, GMP n=9 at 4-5 min). The centre of mass shift turned again to a positive trend at 7 dpf, however GMP-evoked movement was not significantly different from EM values (two way repeated measure ANOVA: $Ftreatment_{1, 117}$=2.150, p =0.160).

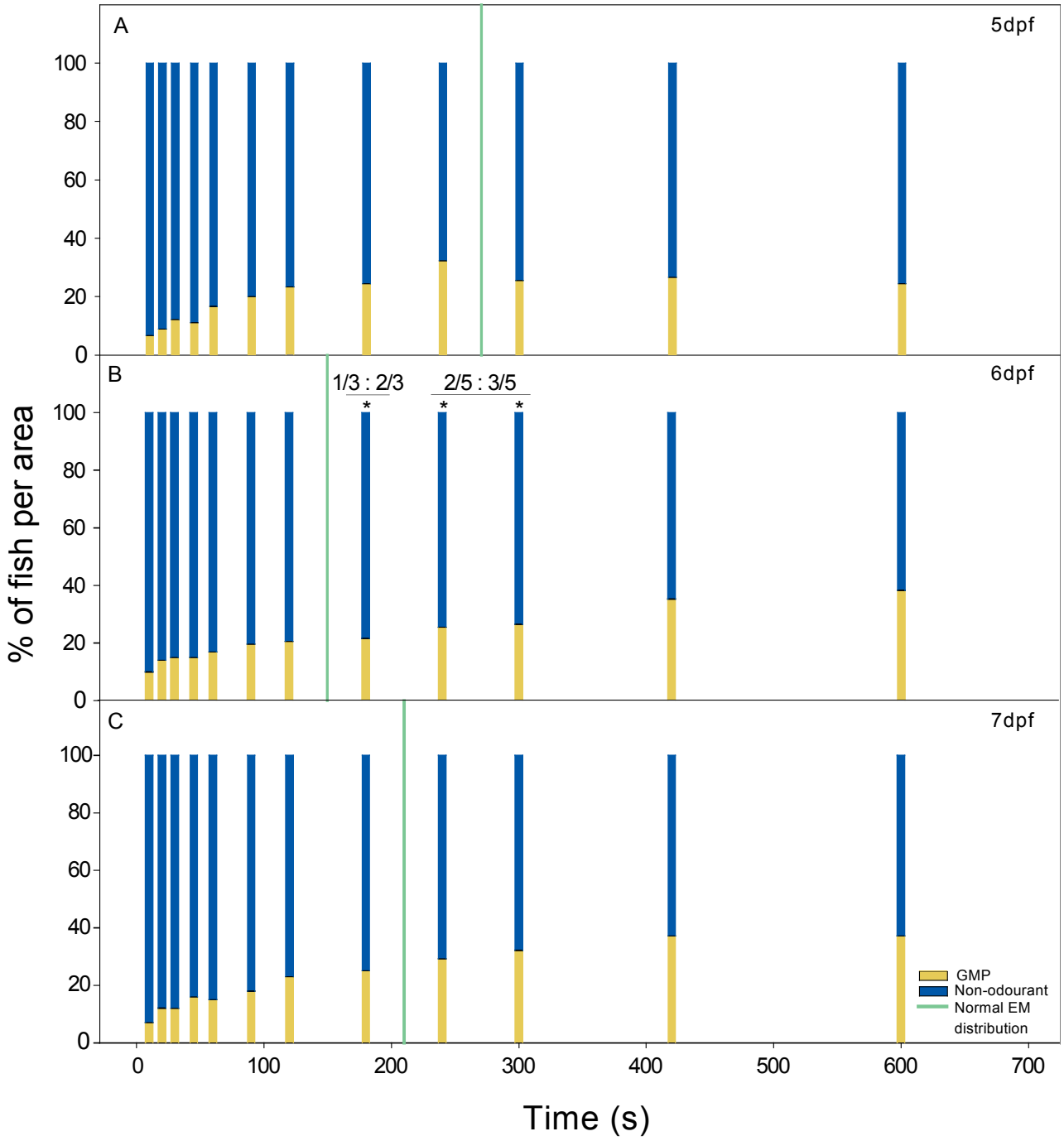

A
5dpf
B
6dpf
C
7dpf
1/3 : 2/3
2/5 : 3/5
*
*
*
% of fish per area
Time (s)
0
100
200
300
400
500
600
700
0
20
40
60
80
100
GMP
Non-odourant
Normal EM distribution

Figure 3.16. Manually scored area occupancy for larval $1x10^{-5}$M GMP trials. A-C show 5, 6 and 7 dpf trials respectively (n=9 trials for 5 dpf and n=10 trials for 6 and 7 dpf; n=9-11 fish per trial). Data is presented as mean percent of fish per area (odourant vs. non-odourant). The green line represents the division between even and skewed EM distributions. Only time points past the green line were analyzed for goodness of fit using heterogeneity chi squared testing. Asterisks denote differences from the expected EM determined odourant: non-odourant distributions. Expected distributions are noted above asterisks.

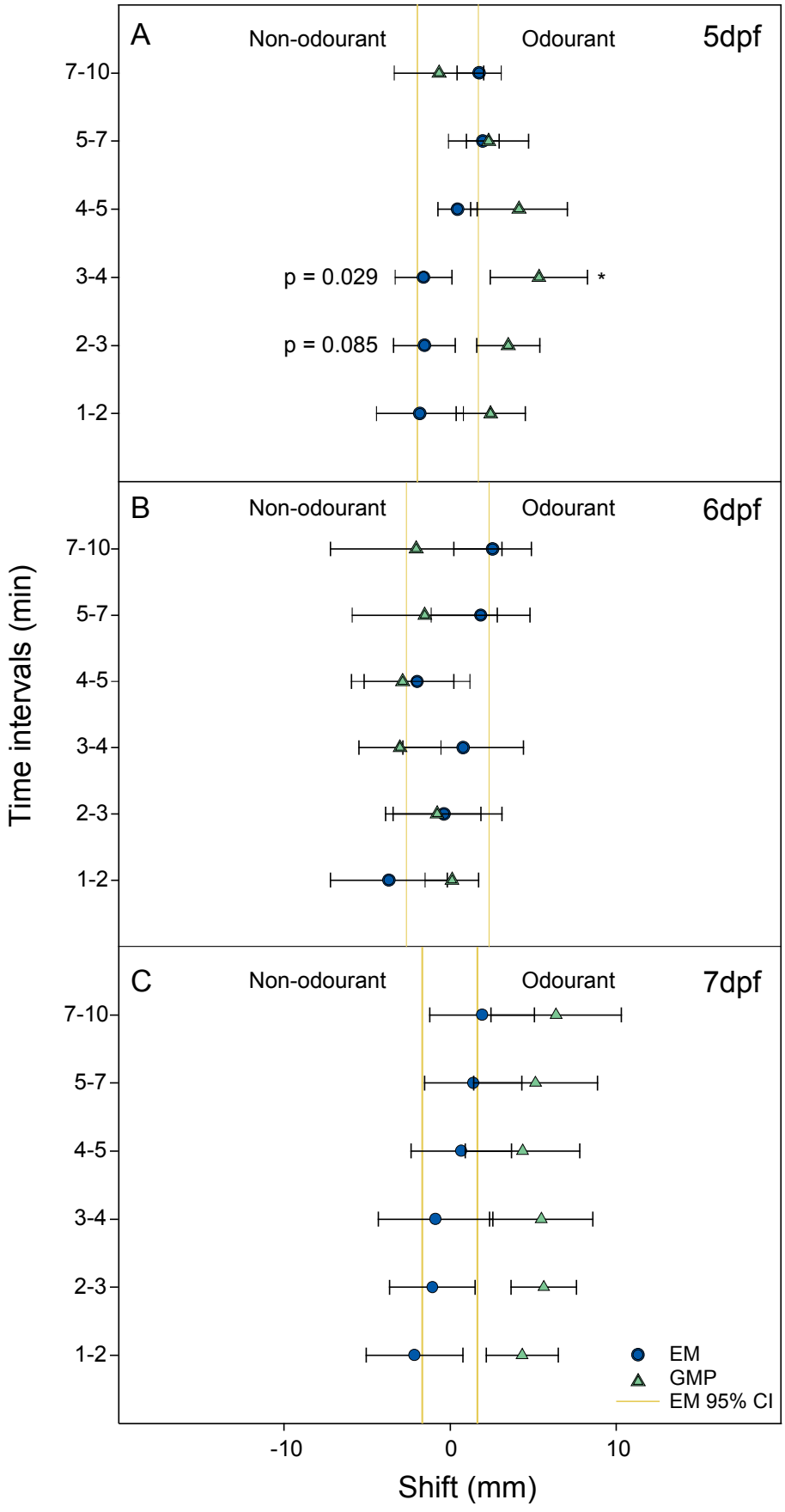

A
Non-odourant
Odourant
5dpf
7-10
5-7
4-5
3-4
p = 0.029
*
2-3
p = 0.085
1-2
B
Non-odourant
Odourant
6dpf
7-10
5-7
4-5
3-4
2-3
1-2
C
Non-odourant
Odourant
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
EM
GMP
EM 95% CI
Time intervals (min)
-10
0
10
Shift (mm)

Figure 3.17. Centre of mass shift (mm) in $1x10^{-5}$M GMP vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10 and n=9 at 4-5 and 7-10 min. GMP n=9 AND n=8 at 1-2 min. B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. GMP n=10 and n=9 at 4-5 min. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. Guanosine n=10. A –C) Data presented as mean shift in centre of mass ± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively. Asterisk denotes significant difference from EM shift.

Larval zebrafish avoidance attraction responses to odourant set 2

Embryo medium

At 5 dpf, odourant set 2 EM controls were comparable to odourant set 1 controls (Figure 3.18; two way repeated measures ANOVA: $Ftreatment_{1, 117}$=0.0408, p =0.842, EM set 1 n=8 1-2 min, EM set 2 n=10). EM trials for odourant set 2 exhibited a right side (positive) apparatus bias at 6 and 7 dpf when compared to the control data from odourant set 1. At 6 dpf, although a two way repeated measures ANOVA showed that treatment did not affect the centre of mass shift, the positive trend could not be dismissed ($Ftreatment_{1, 111}$=2.166, p =0.159; Holm-Sidak 1-2 min p=0.093, EM set 1 n=8 4-5 min and EM set 2 n=10 and n=9 4-5 min). At 7 dpf, although EM treatments were not significantly different overall (two way repeated measures ANOVA: $Ftreatment_{1, 111}$=3.787, p =0.068, EM set 1 n=9 3-4 and 4-5 min, and EM set 2 n=10 and n=9 1-2 min), Holm-Sidak comparisons confirmed a positive bias at 3-4 min (-0.9 ± 3.4 vs. 9.0 ± 2.9mm, p=0.050) and 4-5 min (0.7 ± 3.0 vs. 8.9 ± 2.2mm). EM set 2 5dpf data was unbiased and therefore usable for odourant comparison. Unbiased EM data from odourant set 1 was used for 6

and 7 dpf analysis and was compared to bias corrected odourant set 2 shift values (see Chapter 3 Materials and methods for more details).

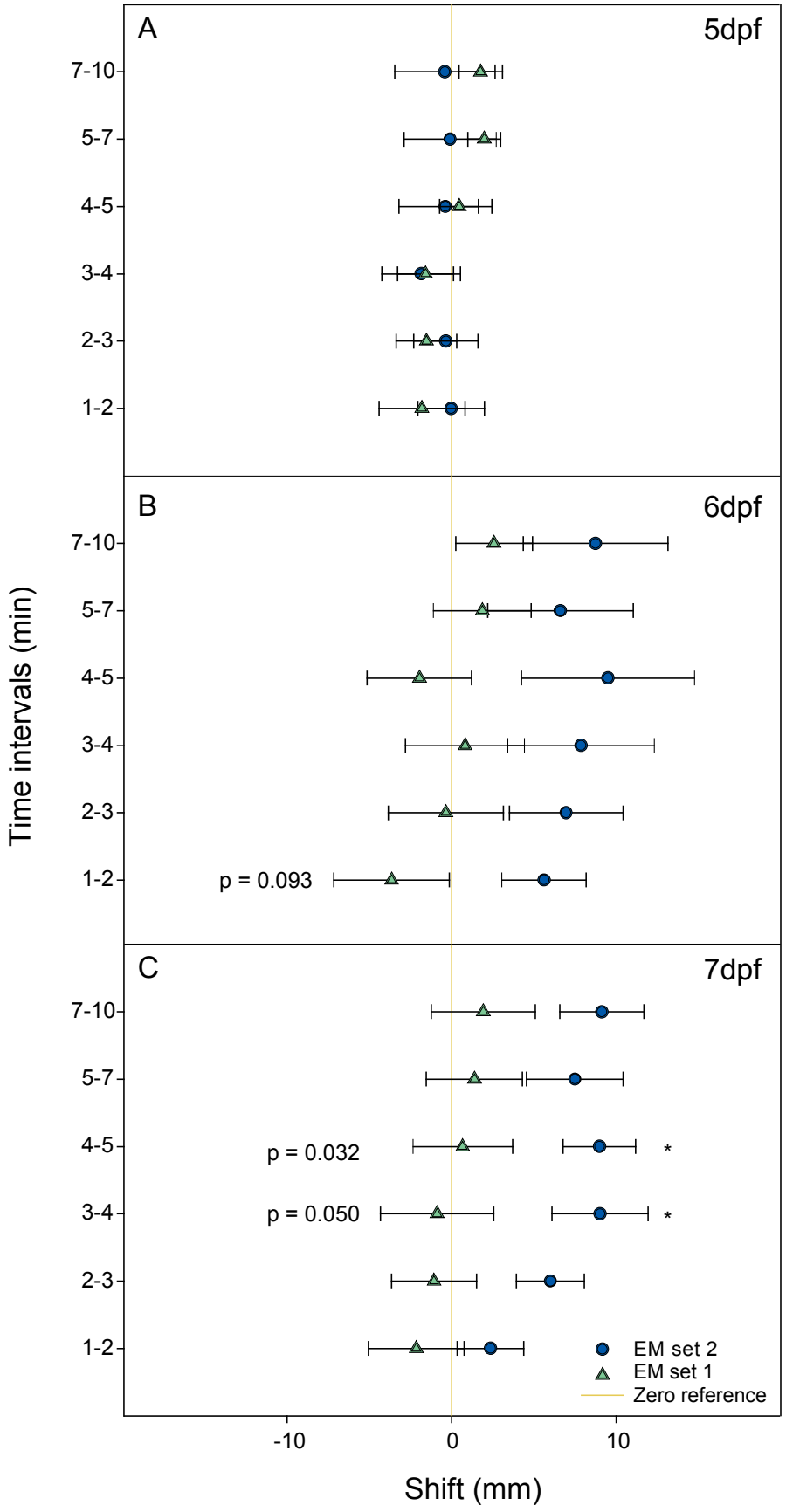

A
5dpf
B
6dpf
C
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
Time intervals (min)
p = 0.093
p = 0.032
p = 0.050
*
*
EM set 2
EM set 1
Zero reference
-10
0
10
Shift (mm)

Figure 3.18. Centre of mass shift (mm) in EM set1 vs. EM set 2 larval avoidance-attraction trials. A) 5 dpf trials: EM set 1 n=10 and n=9 at 4-5 and 7-10 min. EM set 2 n=10. B) 6 dpf trials: EM set 1 n= 9 and at 4-5 min n=8. EM set 2 n=10 and n=9 4-5 min. C) 7 dpf trials: EM set 1 n=10 and n=9 at 3-4 and 4-5 min. EM set 2 M set 2 n=10 and n=9 1-2 min. A –C) Data presented as mean shift in centre of mass ± SEM per time intervals (min). Yellow line represents the zero reference line. Positive and negative shift values indicate movement towards the right and left apparatus sides respectively. Asterisks denote significant differences in centre of mass shift.

Uridine-5'-monophosphate

At 5 dpf, there was no observable difference in centre of mass movement between UMP and EM (Figure 3.19; two way repeated measures ANOVA: $Ftreatment_{1, 113}$=0.0380, p =0.848, UMP n=9). No side bias was observed in UMP 6 dpf trials and therefore values were uncorrected (two way repeated measures ANOVA: $Fside_{1, 59}$=0.233, p =0.642, n=10). There was no difference in centre of mass shift between UMP and EM trials in 6 dpf larvae (two way repeated measures ANOVA: $Ftreatment_{1, 112}$=0.324, p =0.577). A right side bias was observed for 7 dpf UMP trials which increased with time (two way repeated measures ANOVA: $Fside_{1, 59}$=11.431, p =0.010; $Fside\ x\ time_{5, 59}$=7.534, p <0.001, UMP n=10). Bias corrected 7 dpf UMP centre of mass movement was not different from control trials (two way repeated measures ANOVA: $Ftreatment_{1, 117}$=0.00881, p =0.926). While purine containing monophosphates (AMP and GMP) induced behavioural responses, pyrimidine containing UMP did not.

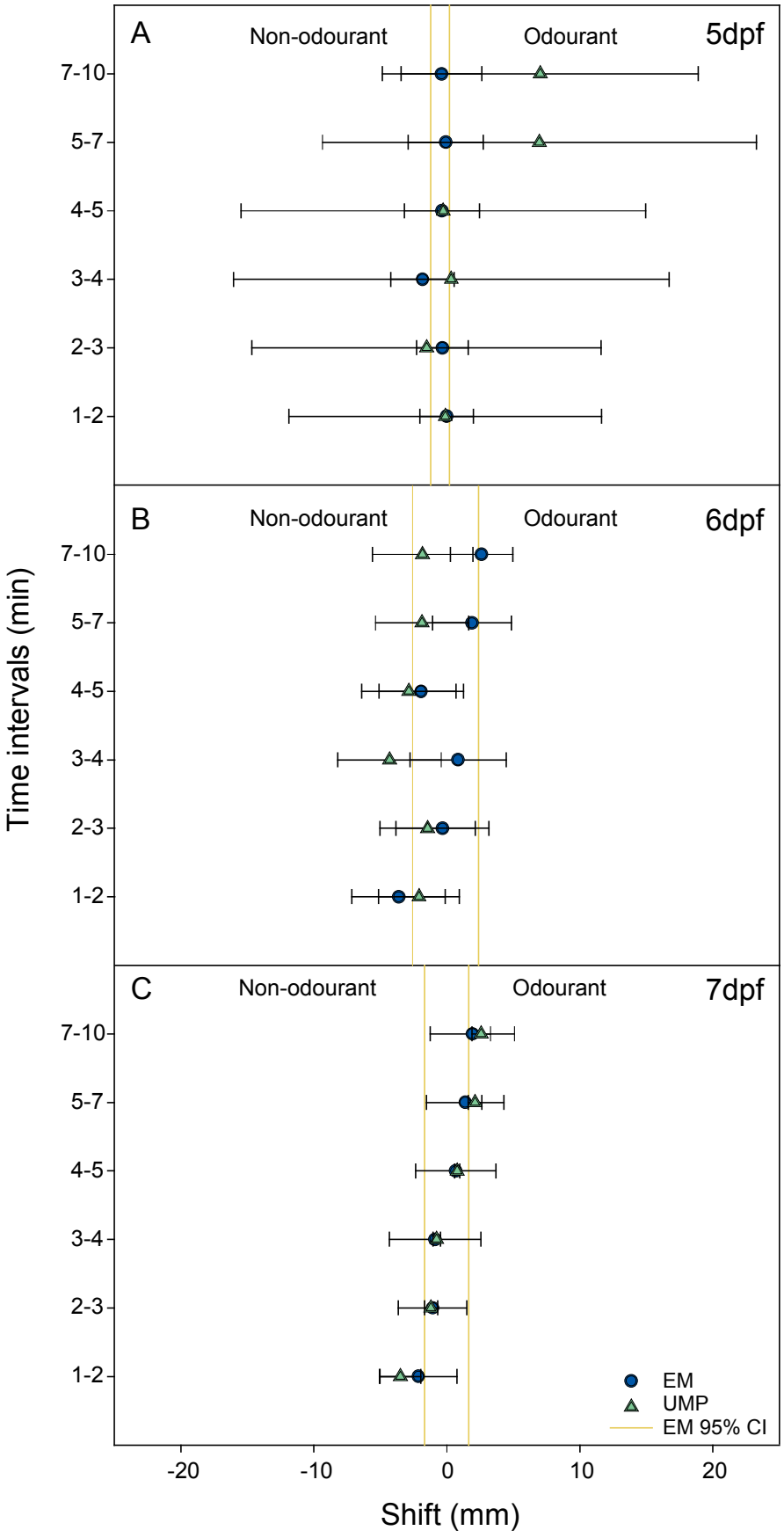

A
Non-odourant
Odourant
5dpf
B
Non-odourant
Odourant
6dpf
C
Non-odourant
Odourant
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
Time intervals (min)
-20
-10
0
10
20
Shift (mm)
EM
UMP
EM 95% CI

Figure 3.19. Centre of mass shift (mm) in $1x10^{-5}$M UMP vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10. UMP n=9. B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. UMP n=10. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. UMP n=10. UMP data was bias corrected. A –C) Data presented as mean shift in centre of mass ± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively.

Inosine-5'-monphosphate

There was no observable difference in centre of mass shift in EM compared to IMP 5 dpf trials (Figure 3.20; two way repeated measures ANOVA: $Ftreatment_{1, 118}$=0.111, p =0.742, IMP n=10, n=9 at 4-5 min). Side bias was not a factor in 6 dpf IMP trials (two way repeated measures ANOVA: $Fside_{1, 59}$=0.643, p =0.446, IMP n=10). Odourant treatment did not affect centre of mass shift at 6 dpf however, the attraction trend in IMP trials did decrease with increasing time (two way repeated measures ANOVA: $Ftreatment_{1, 112}$=0.135, p =0.718; $Ftreatment\ x\ time_{1, 112}$=3.912, p =0.003). The centre of mass shift towards IMP was noticeable at 1-2 min (Holm-Sidak: p= 0.076). 7 dpf IMP trials also showed no side bias (two way repeated measures ANOVA: $Fside_{1, 58}$=0.536, p =0.485, IMP n=10, n=9 at 1-2 min). Centre of mass shift with IMP treatment was no different from EM trials (two way repeated measures ANOVA: $Ftreatment_{1, 116}$=0.490, p =0.493).

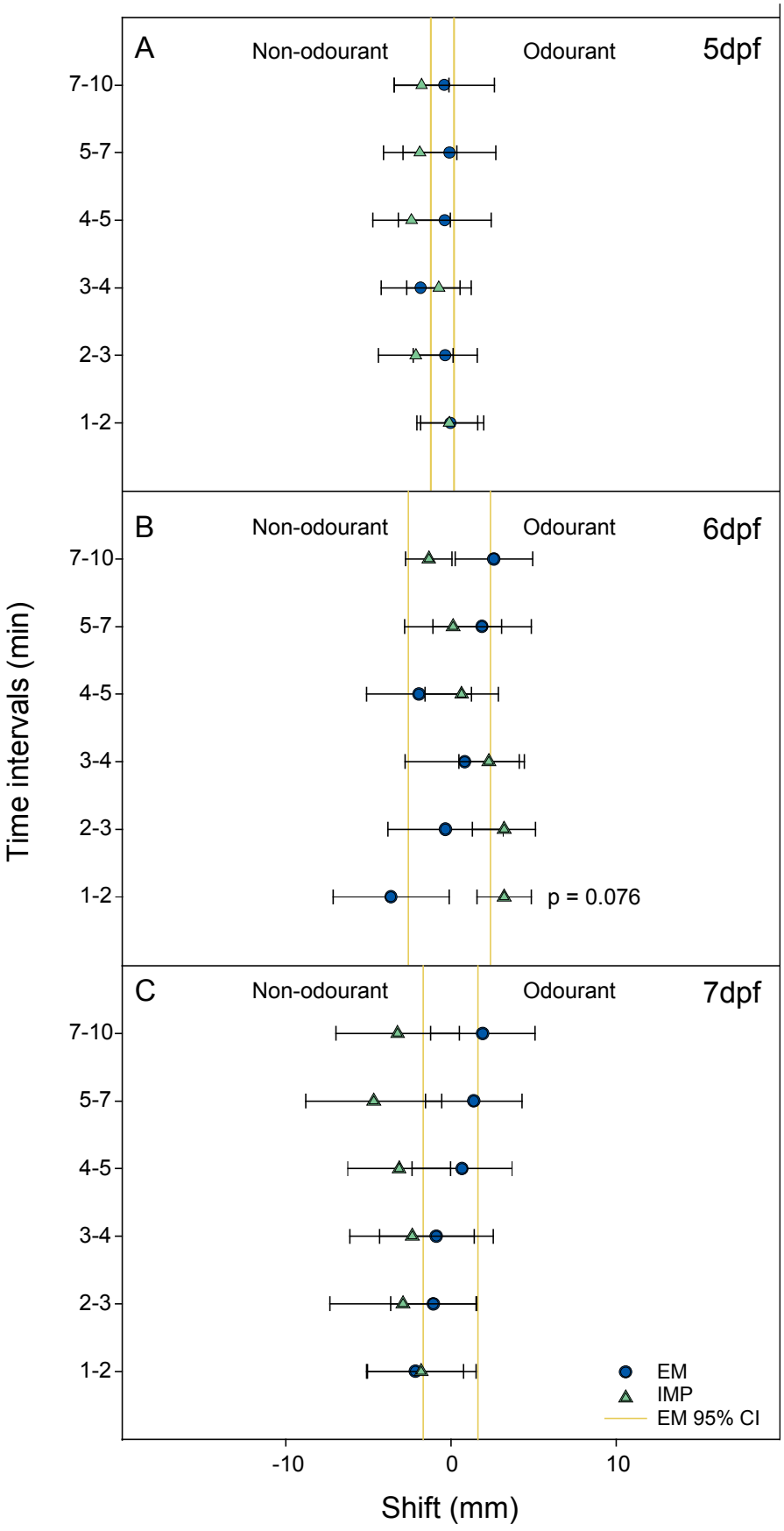

A
Non-odourant
Odourant
5dpf
B
Non-odourant
Odourant
6dpf
p = 0.076
C
Non-odourant
Odourant
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
Time intervals (min)
EM
IMP
EM 95% CI
-10
0
10
Shift (mm)

Figure 3.20. Centre of mass shift (mm) in $1x10^{-5}$M IMP vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10. IMP n=10 and n=9 at 4-5min. B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. IMP n=10. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. IMP n=10 and n=9 at 1-2 min. A –C) Data presented as mean shift in centre of mass ± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively.

Cytidine-5'-monophosphate

At 5 dpf, CMP centre of mass shifts did not differ from EM trials (Figure 3.21; two way repeated measures ANOVA: $Ftreatment_{1, 117}$=0.0367, p =0.850, CMP n=10, n=9 at 1-2 min and 4-5 min). At 6 dpf, CMP trials showed no side bias and treatment did not affect centre of mass shift (two way repeated measures ANOVA: $Fside_{1, 59}$=1.295, p =0.288; F$treatment_{1, 112}$=0.179, p =0.678, CMP n=10). CMP 7 dpf trials, however, showed a strong right side bias (two way repeated measures ANOVA: $Fside_{1, 58}$=9.146, p =0.016, CMP n=10, n=9 at 4-5 min). Bias corrected centre of mass values were no different from EM-evoked shifts (two way repeated measures ANOVA: $Ftreatment_{1, 116}$=0.110, p =0.745). Overall, another pyrimidine containing nucleotide was not behaviourally active at any age.

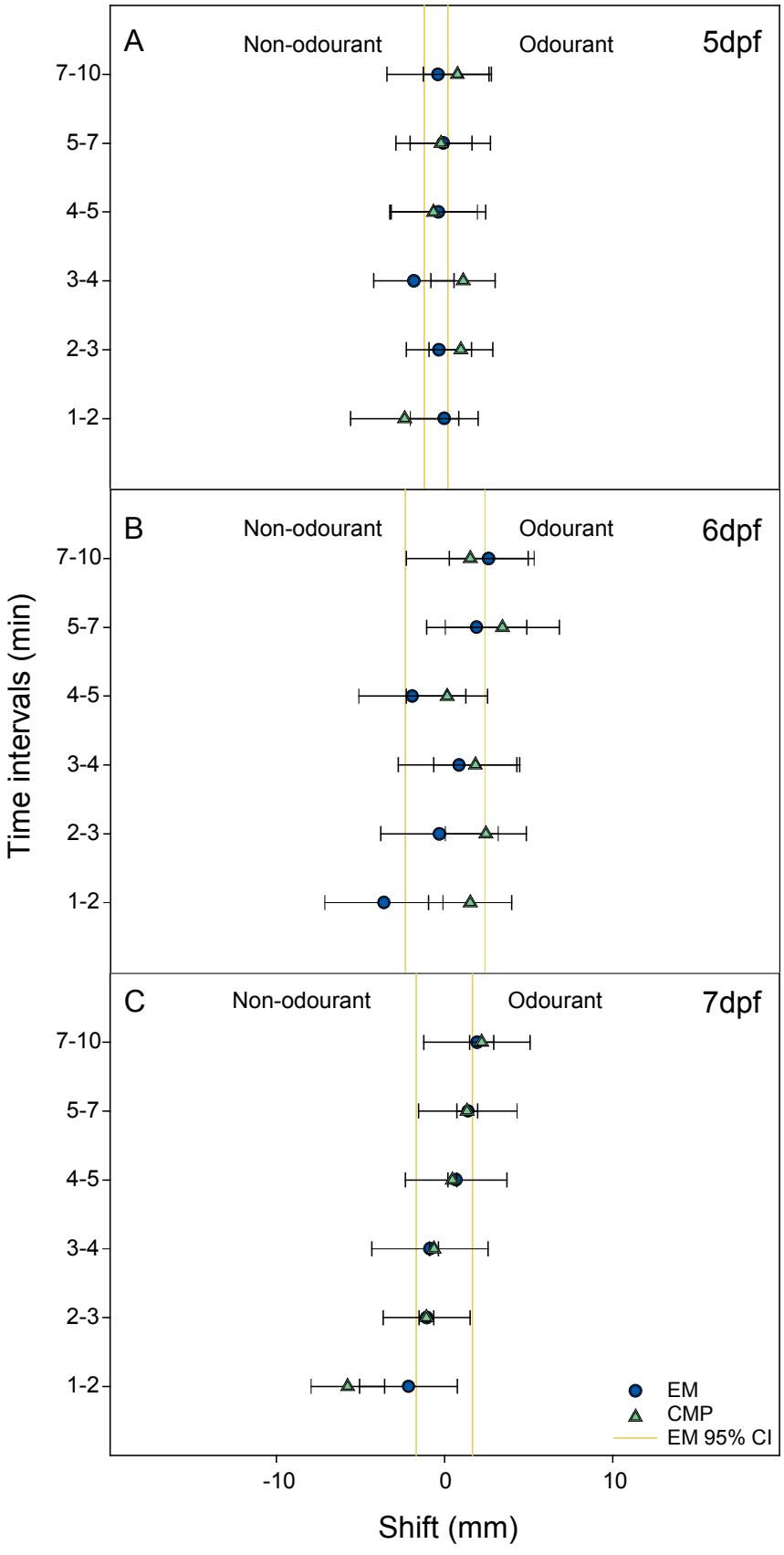

A
Non-odourant
Odourant
5dpf
B
Non-odourant
Odourant
6dpf
C
Non-odourant
Odourant
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
Time intervals (min)
EM
CMP
EM 95% CI
-10
0
10
Shift (mm)

Figure 3.21. Centre of mass shift (mm) in $1x10^{-5}$M CMP vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10. CMP n=10 and n=9 at 1-2 and 4-5 min. B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. CMP n=10. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. CMP n=10 and n=9 at 4-5 min. CMP data was bias corrected. A –C) Data presented as mean shift in centre of mass ± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively.

Hypoxanthine

Hypoxanthine did not evoke an avoidance-attraction response in 5 dpf fish (Figure 3.22; two way repeated measures ANOVA: $Ftreatment_{1, 118}$=0.0162, p =0.900, hypoxanthine n=10 and n=9 at 2-3 min). At 6 dpf, hypoxanthine trials had a bias at select time points (two way repeated measures ANOVA: $Fside_{1, 59}$=4.562, p =0.065, Holm-Sidak: p =0.085, 0.043, 0.066 and 0.023 for 3-4, 4-5, 5-7 and 7-10 min respectively, n=10). Corrected hypoxanthine centre of mass shifts showed no difference in area use compared to EM trials (two way repeated measures ANOVA: $Ftreatment_{1, 112}$=0.000, p =0.999). No side bias was observed for 7 dpf hypoxanthine trials (two way repeated measures ANOVA: $Fside_{1, 53}$=1.412, p =0.273, n=9). Hypoxanthine provoked a shift towards the non-odourant zone, particularly during 4-5 min, which indicated a short avoidance response, but overall odourant treatment did not affect area use (two way repeated measures ANOVA: $Ftreatment_{1, 111}$=1.642, p =0.217, Holm-Sidak 4-5 min: p =0.096). Hypoxanthine differs from the behaviour evoking compound adenine by one functional group, yet the small change resulted in hypoxanthine induced avoidance behaviour at 7 dpf instead of 5 dpf.

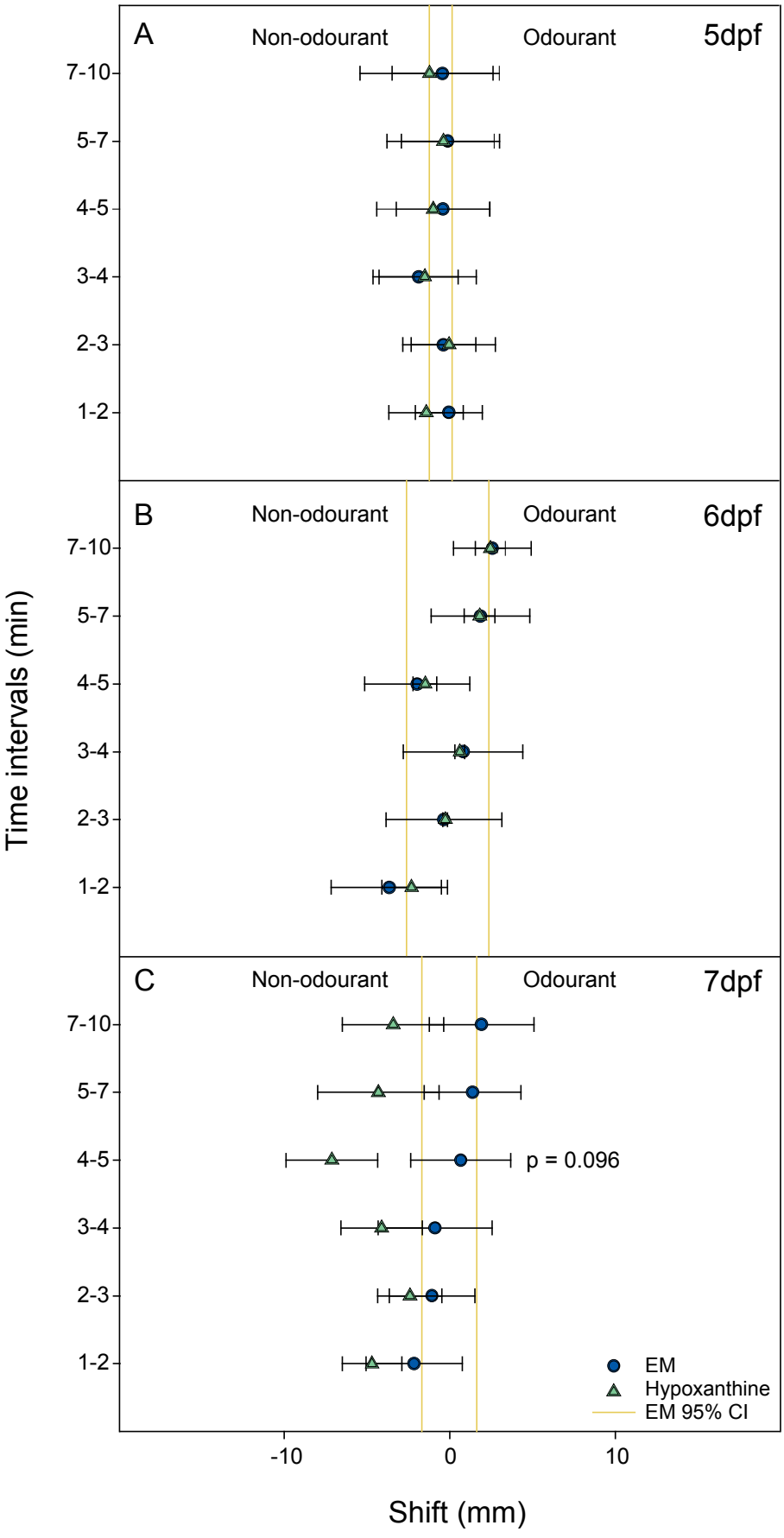

A
Non-odourant
Odourant
5dpf
B
Non-odourant
Odourant
6dpf
C
Non-odourant
Odourant
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
Time intervals (min)
p = 0.096
EM
Hypoxanthine
EM 95% CI
-10
0
10
Shift (mm)

Figure 3.22. Centre of mass shift (mm) in $1x10^{-5}$M hypoxanthine vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10. Hypoxanthine n=10 and n=9 at 2-3 min. B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. Hypoxanthine n=10. Hypoxanthine data was bias corrected. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. Hypoxanthine n=9. A –C) Data presented as mean shift in Centre of mass ± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively.

Hypoxanthine-3-N-oxide

H3NO, which differs by one functional group from the effective nucleobase hypoxanthine, did not have different shifts in centre of mass compared to EM at 5 dpf (Figure 3.23; two way repeated measures ANOVA: $Ftreatment_{1, 118}$=0.524, p =0.497, rank transformed, H3NO n=10 and n=9 at 2-3 min). 6 dpf trials did reveal a centre of mass shift bias for the right side which increased with time (two way repeated measures ANOVA: $Fside_{1, 53}$=11.796, p =0.011; $Fside\ x\ time_{5, 53}$=2.545, p =0.046, n=9). Bias corrected 6 dpf H3NO values showed no difference in centre of mass shift compared to EM trials (two way repeated measures ANOVA: $Ftreatment_{1, 106}$=0.000, p =0.995). No right side bias was observed in 7 dpf H3NO trials and there was no difference in odourant vs. EM centre of mass movement (two way repeated measures ANOVA: $Fside_{1, 52}$=0.022, p =0.886, $Ftreatment_{1, 110}$=0.101, p =0.755, H3NO n= 9 and n=8 4-5 min).

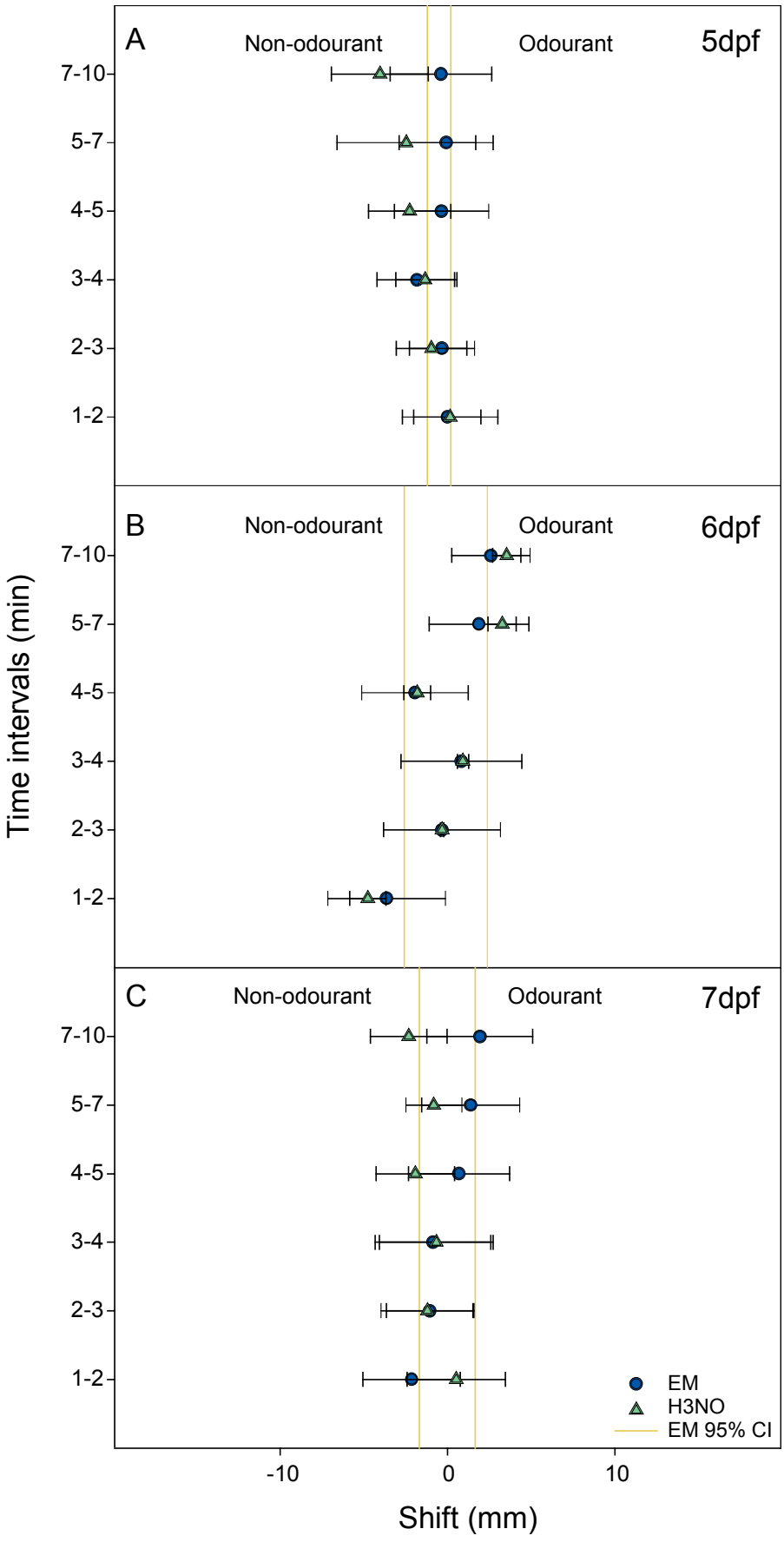

A
Non-odourant
Odourant
5dpf
B
Non-odourant
Odourant
6dpf
C
Non-odourant
Odourant
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
Time intervals (min)
-10
0
10
Shift (mm)
EM
H3NO
EM 95% CI

Figure 3.23. Centre of mass shift (mm) in $5x10^{-9}$M H3NO vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10. H3NO n=10 and n=9 at 2-3 min. B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. H3NO n=9. H3NO data was bias corrected. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. H3NO n= 9 and n=8 4-5 min. A –C) Data presented as mean shift in centre of mass ± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones

Nucleotide mixture

The NT mixture, containing both purine and pyrimidine nucleotides, did not affect centre of mass movement at 5 dpf (Figure 3.24; two way repeated measures ANOVA: $Ftreatment_{1, 119}$=0.923, p =0.350, NT mix n=10). At 6 dpf, there was no apparatus side bias in NT mixture trials and no difference in centre of mass shift compared to controls (two way repeated measures ANOVA: $Fside_{1, 53}$=1.290, p =0.293, $Ftreatment_{1, 106}$=0.791, p =0.387, NT mix n= 9). Similarly, in 7 dpf NT mixture trials, no side bias or significant shift in centre of mass were found (two way repeated measures ANOVA: $Fside_{1, 57}$=2.610, p =0.145, $Ftreatment_{1, 115}$=0.200, p =0.660, NT mix n= 10 and n=9 at 2-3 and 3-4 min).

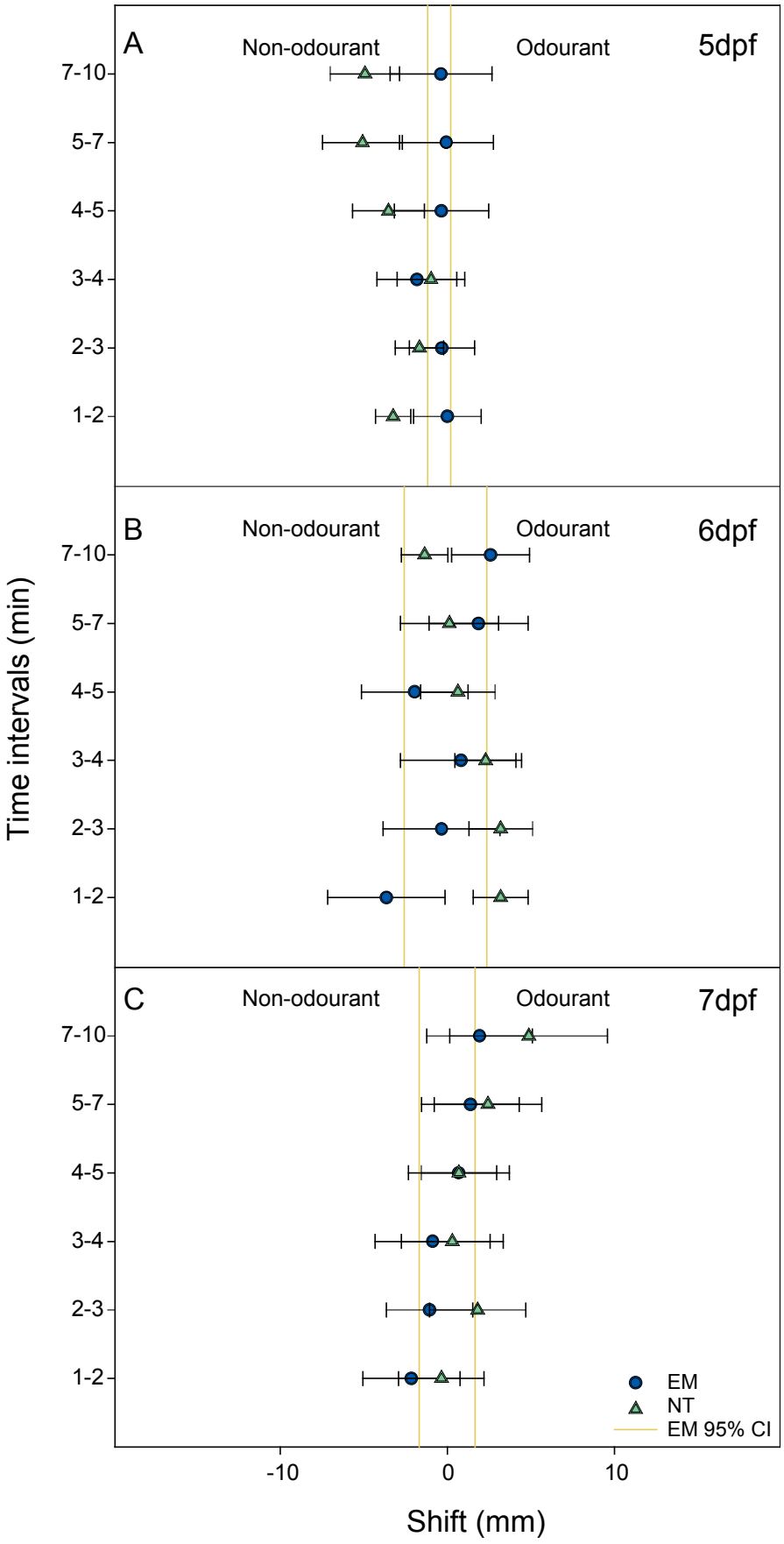

A
Non-odourant
Odourant
5dpf
B
Non-odourant
Odourant
6dpf
C
Non-odourant
Odourant
7dpf
7-10
5-7
4-5
3-4
2-3
1-2
Time intervals (min)
-10
0
10
Shift (mm)
EM
NT
EM 95% CI

Figure 3.24. Centre of mass shift (mm) in $4x10^{-5}$M NT mixture vs. EM larval avoidance-attraction trials. A) 5 dpf trials: EM n=10. NT mix n=10. B) 6 dpf trials: EM n= 9 and at 4-5 min n=8. NT mix n= 9. C) 7 dpf trials: EM n=10 and n=9 at 3-4 and 4-5 min. NT mix n= 10 and n=9 at 2-3 and 3-4 min. A –C) Data presented as mean shift in centre of mass ± SEM per time intervals (min). Yellow lines represent the 95% confidence intervals (CI) for EM. Positive and negative shift values indicate movement towards odourant and non-odourant zones respectively.

Summary

Of the nucleobase compounds tested, there was diversity between the types of responses (avoidance or attraction) observed. This diversity in response type did not follow a trend relating to compound structure or developmental age. All adenine containing compounds evoked changes in behaviour. Deviations from the nucleobase adenine, depending on the degree of change, resulted in changes in behaviour type and age of behavioural response. Notably, all purine (double ring nucleobases) containing nucleotides (AMP, ATP, GMP and IMP) induced behavioural responses, while pyrimidine (single ring nucleobases) containing nucleotides did not evoke behavioural responses at any age.

Larval zebrafish avoidance-attraction responses in repeat trials

Embryo medium repeat trials

Repeat trials showed observable biases towards the right side of the apparatus (Figure 3.25). The right side bias was at its maximum at 6 dpf trials where it notably differed from 7 dpf centre of mass movement at 1-2 and 2-3 min (two way repeated measures ANOVA: $Fage_{2, 175}$=2.613, p

=0.101, Holm-Sidak p= 0.0530 and 0.0930, n=10 each 5 dpf: n=9 at 1-2 min and n=8 at 2-3 min, 6 dpf: n=9 at 4-5 min). It is interesting that centre of mass movement at 5 and 7 dpf did not differ, but the bias was exacerbated at 6 dpf and reduced at 7 dpf. Overall, the overwhelming right side bias impairs deeper interpretation of the results.

Food extract repeat trials

Although repeat FE trials showed an attraction trend at 5 dpf, an avoidance trend at 6 dpf and a more neutral trend at 7 dpf, overall movement at each age did not differ significantly (Figure 3.26; two way repeated measures ANOVA: $Fage_{2, 160}$=0.444, p =0.649, n= 9 each, 5 dpf: n=8 at 1-2 min).

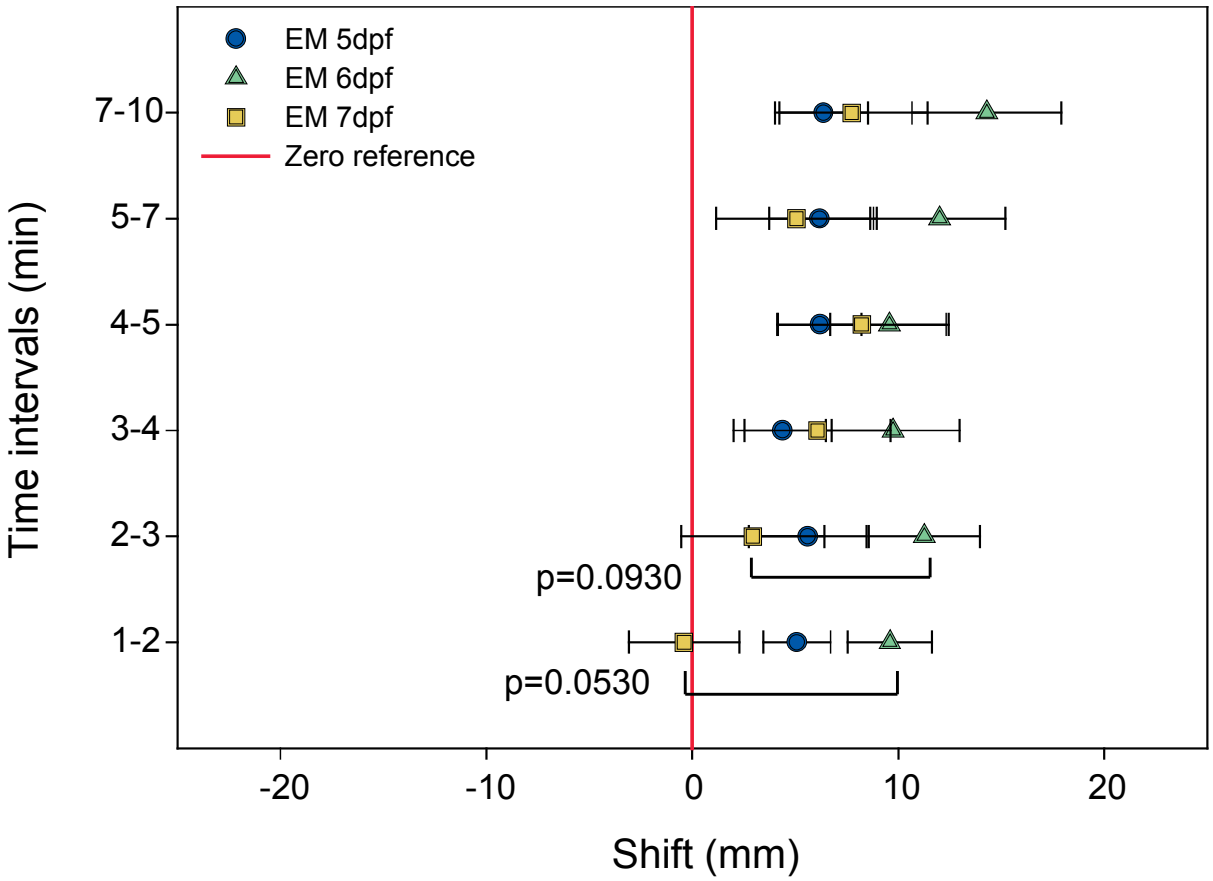


Figure 3.25. Centre of mass shift (mm) in repeat 5, 6 and 7 dpf EM larval avoidance-attraction trials. N=10 for each age. For 5 dpf, n=9 at 1-2 min and n=8 at 2-3 min. For 6 dpf, n=9 at 4-5 min. Data presented as mean shift in centre of mass ± SEM per time intervals (min). Red line marks zero reference point. Positive and negative shift values indicate movement towards the right and left apparatus sides respectively.

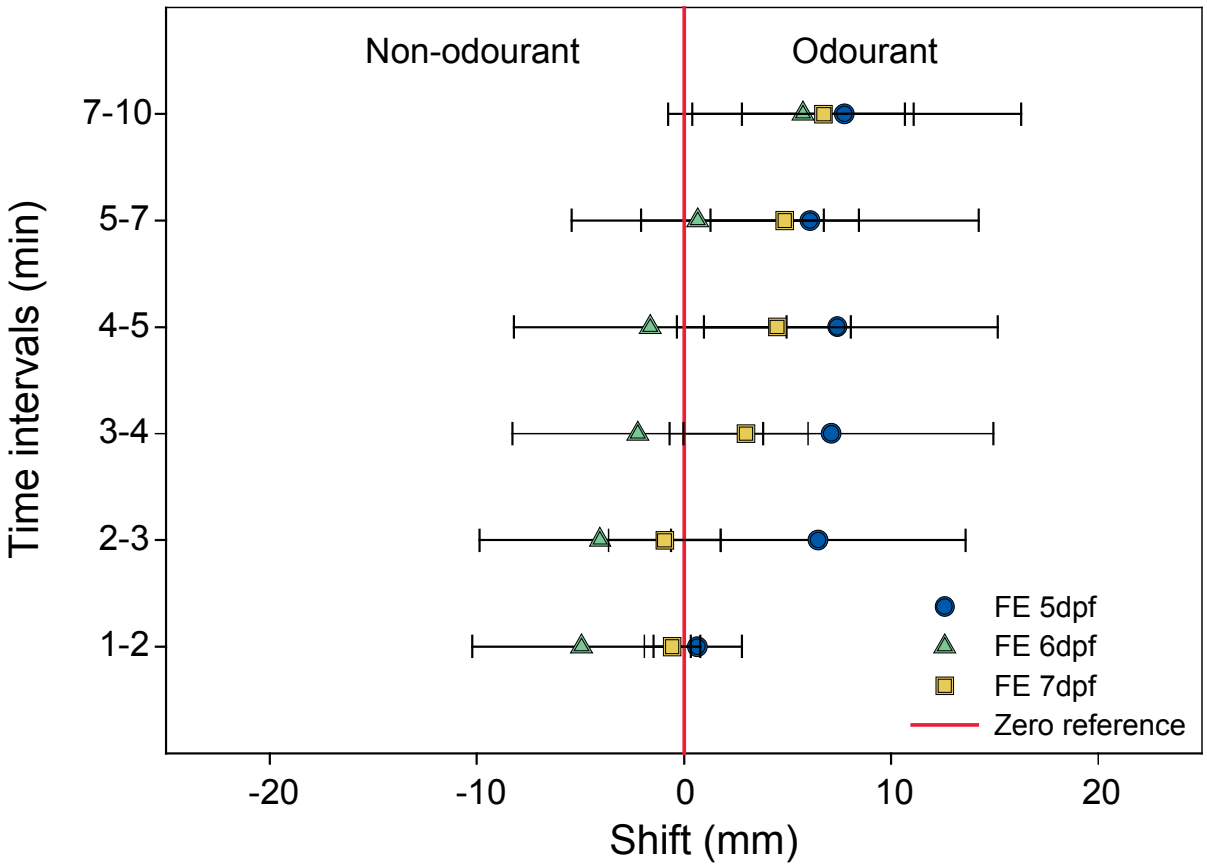


Figure 3.26. Centre of mass shift (mm) in repeat 5, 6 and 7 dpf 1:100 FE larval avoidance-attraction trials. N=9 for each age. For 5 dpf, n=8 at 1-2 min. Data presented as mean shift in centre of mass ± SEM per time intervals (min). Red line marks zero reference point. Positive and negative shift values indicate movement towards the odourant and non-odourant zones respectively.

Adenosine-5'-monophosphate repeat trials

In repeated AMP trials a negative (avoidance) trend for fish tested at both 5 and 6 dpf compared to a more neutral trend at 7 dpf was noted, however the differences were not significant (Figure 3.27; two way repeated measures ANOVA: $Fage_{2, 160}$=0.498, p =0.617, n= 9 per age, 6 dpf: n=8 at 2-3 min). Comparing AMP 7 dpf results from set 1 (naïve exposures) to the AMP 7 dpf repeated exposures, naïve exposure centres of mass had a trend supporting a greater shift away from AMP (Figure 3.28; two way repeated measures ANOVA: $Fexposures_{1, 105}$=2.778, p =0.115). This was specifically noted at 4-5 min where naïve exposure centre of mass shift is -6.4 ± 3.0mm vs. the repeat exposure centre of mass shift of 1.8 ± 3.7mm Holm- Sidak: p =0.087). Results should be interpreted cautiously due to observed right side bias in repeat EM trials and inability to correct for possible biases in these odourant trials.

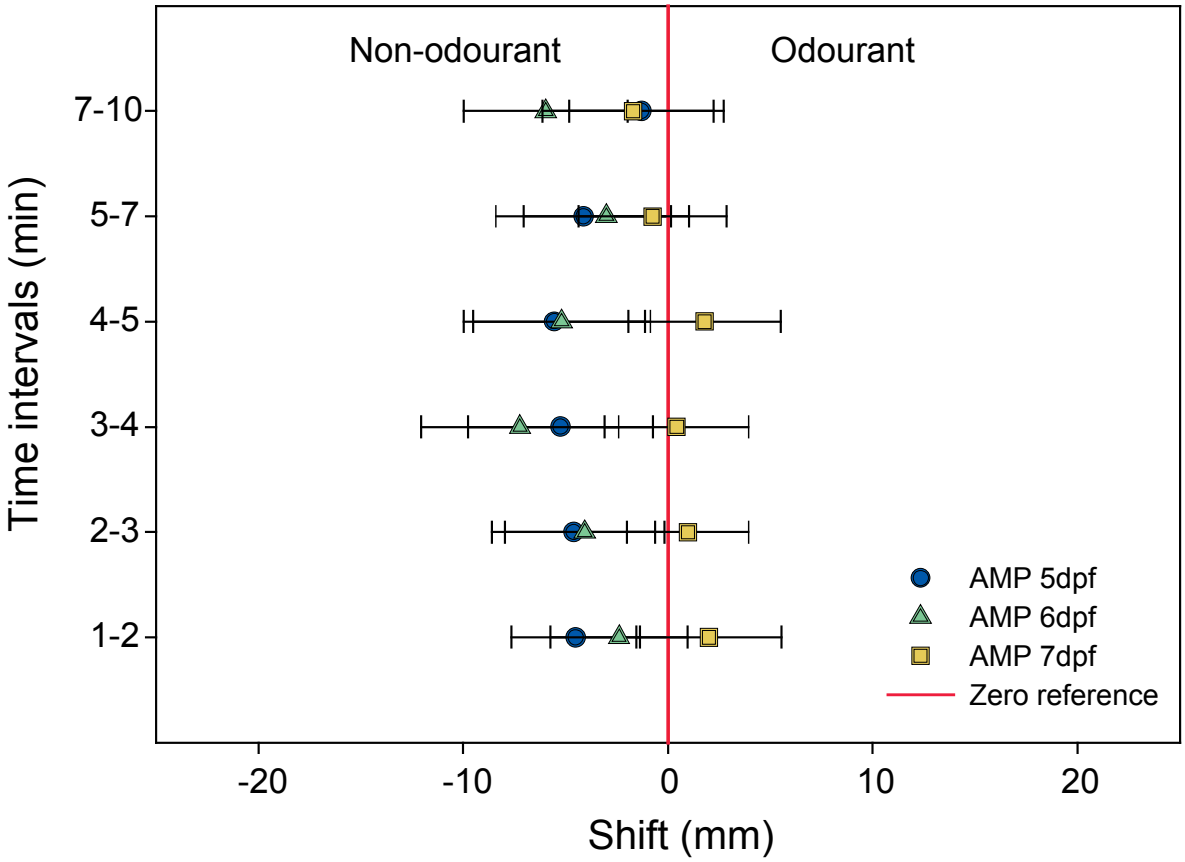


Figure 3.27. Centre of mass shift (mm) in repeat 5, 6 and 7 dpf $1x10^{-5}$M AMP larval avoidance-attraction trials. N=9 for each age. For 5 dpf, n=8 at 1-2 min. Data presented as mean shift in centre of mass ± SEM per time intervals (min). Red line marks zero reference point. Positive and negative shift values indicate movement towards the odourant and non-odourant zones respectively.

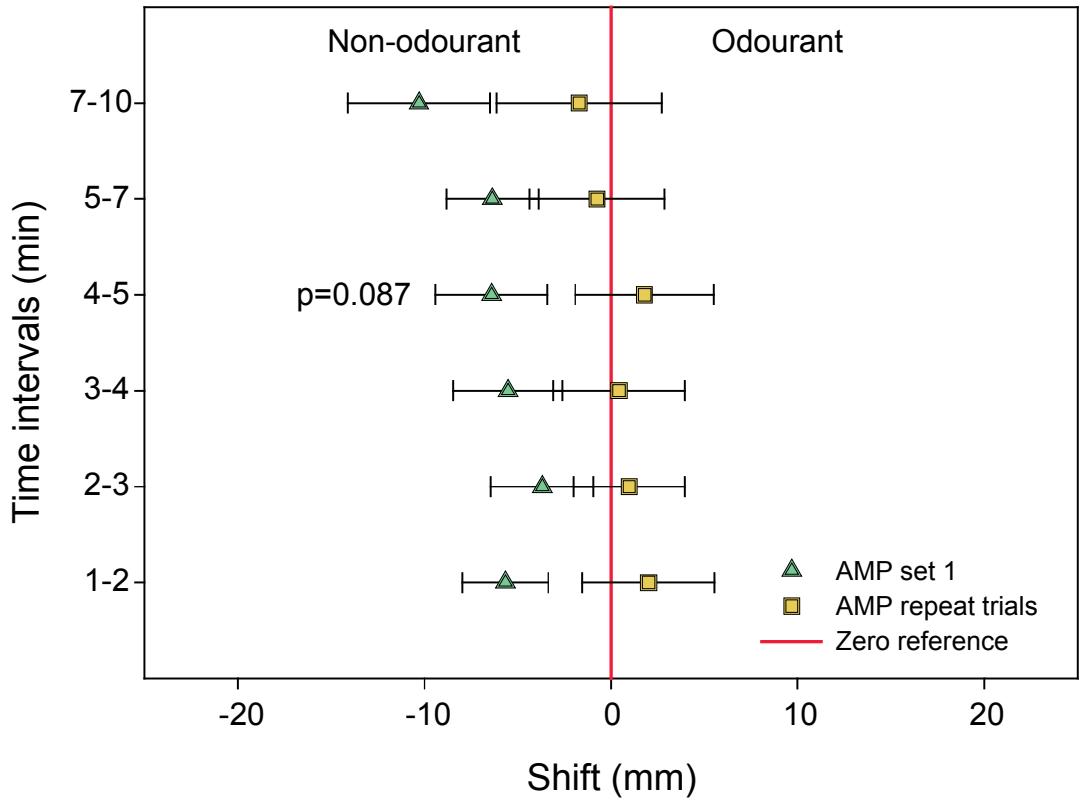


Figure 3.28. Centre of mass shift (mm) in repeat 7 dpf vs. naïve 7 dpf $1x10^{-5}$M AMP larval avoidance-attraction trials. N=9 for each treatment and for naïve AMP trials n=8 at 1-2 and 7-10 min. Data is shown as mean shift in centre of mass ± SEM per time intervals (min). Red line marks zero reference point. Positive and negative shift values indicate movement towards the odourant and non-odourant zones respectively.

Discussion

Overall, the results of this study demonstrate that both larval and adult zebrafish behaviourally respond to nucleobase compounds, most of which are novel findings. Larvae responded primarily with avoidance, while adult zebrafish demonstrated behaviours akin to an L-alanine reaction (a food odourant). Expanded investigation of larval avoidance-attraction videos has shown centre of mass data as superior to that obtained from manual scoring. Data also showed variable responses depending on odourant structure, fish age and naivety, however additional experimentation is required to better characterize any trends. Some odourant responses (set 2 and repeat trial experiments) should be interpreted with caution and repeated due to the prominent bias for the right side of the apparatus. Nevertheless, this study shed further light on the dynamic nature of the early developing olfactory system as shown through behavioural responses to nucleobase compounds.

Nucleobase compounds evoked olfactory behaviours in zebrafish

Adult zebrafish respond to adenosine

Adult zebrafish exposed to adenosine had a similar behavioural response to that of L-alanine, which is a well-established food odourant in zebrafish [38, 51]. Both odourants evoked an increase in bottom dwelling behaviour and this finding was enhanced by performing spike analysis. This is a valuable tool for examining behavioural responses in zebrafish because it aligns responses between individuals that occurred within different time bins. Without accounting for subtle variations between response times, changes in activity are often difficult to detect. For L-alanine responses, an increase in bottom dwelling behaviour post odourant exposure was expected as test subjects had been raised and conditioned to sinking food. In

nature, wild strains of zebrafish are more likely to feed on insects that are higher in the water column, however the laboratory rearing conditions of my test subjects have altered this behaviour [87]. Subsequently, increased foraging in the bottom half of the tank was expected for feeding stimulants. Despite the mirrored behaviour in adenosine exposed fish, it is important to note that bottom dwelling can also be indicative of anxious behaviour similar to that seen in alarm compound exposure [88]. No change in average velocity among treatments originally suggested the absence of fear related dashing or freezing movements, however further scrutiny revealed the possibility that these activities could potentially negate each other. In the latter scenario, an alarm response may have occurred without a discernible difference in average velocity. The precise nature behind the adenosine triggered bottom dwelling cannot be elucidated without examining additional metrics such as frequency of >90° turns (feeding) and erratic vs. immobile events (alarm) [37, 88]. While the associated motivation in the response to adenosine should be investigated in future, the immediate purpose of this experiment was simply to determine whether adult zebrafish alter their behaviour in any capacity following adenosine exposure. Due to species variation in other studies, it was important to confirm nucleobase detection and behavioural response in a fully developed zebrafish before investigating these same questions in the larvae [35, 39]. In this respect, this experiment was largely preliminary and successfully supported the rationale for larval studies.

Larval behavioural responses are dependent on the analytical method

As expected, larvae exposed to nucleobase compounds, exhibited shifts in area occupancy indicative of avoidance or attraction responses which confirmed that select odourants are in fact are behaviourally active. To examine data reliability between analysis methods and further clarify results, odourant set 1 was both manually scored and analyzed via videogram creation.

Manual scoring showed several avoidance responses at 6 dpf, which were not observed in centre of mass data leading to suspicions concerning the validity of these results. Videogram analysis supported both the even distribution of control treated fish and several robust avoidance responses observed by manual scoring. Centre of mass data also made several observations possible that were not evident by manual scoring analysis. It is important to note that some attraction responses seen through videogram analysis were not detected via manual scoring.

Congruent results found between videogram and manual analysis of EM and several odourants further validated the avoidance-attraction methodology. Ultimately, I deemed centre of mass data more reliable because it encompassed fish movement in ~120 frames per minute compared to the single frame snap-shot used in manual scoring. By encompassing more frames, videogram analysis was more representative of area occupation without the excessive work and expense that tracking software necessitates. Additionally, videogram analysis examined movement towards or away from the odourant side. Measuring global shift instead of individual fish position removes the need for the distinct odourant vs. non-odourant zones which are vulnerable to false negatives (see Chapter 2). Subsequently, videogram analysis was used for all other avoidance-attraction experiments.

Olfactory responses are dependent on nucleobase structure

Based on behavioural results in several species, it was expected that compounds containing phosphate(s) would elicit an attractive response, while solo nucleobase rings would provoke an avoidance response similar to H3NO. The results of this study match the hypothesis in part, but are in fact more complicated than anticipated (Table 3.1; summary figure Appendix C).

Firstly, as expected, nucleobases adenine and hypoxanthine evoked avoidance responses, albeit at different ages. This is a particularly interesting result because in fathead minnow and finescale dace experiments, compounds that lacked a nitrogen-oxide functional group, including hypoxanthine, failed to elicit alarm responses [41]. These findings contradict the theory that hypoxanthine is not behaviourally active and support my theory that solo nucleobases, due to similarity to H3NO, have a negative association in zebrafish. It was also surprising that H3NO, a potent alarm inducing compound (now identified as effective in larvae; see Chapter 2), did not provoke an avoidance response. This suggests that larvae respond instead to H3NO with changes in movement, such as distance travelled and maximum velocity (identified in Chapter 2), which are not measured in avoidance-attraction testing. Notably, the previous study that examined hypoxanthine and other purines limited their analysis to typical alarm behaviours such as changes in area use and shoaling index; avoidance-attraction was not tested [41]. The differences here are similar to other nucleobase studies where some odourants evoked attraction or activity, but not both behaviours [39]. The different findings between the nucleobase response and the type of behavioural test used reiterate the importance of testing multiple metrics when determining an odourant's behavioural efficacy. In the present study, the results suggest that while the nitrogen-oxide group is related to an *alarm* response, the basic purine ring structure evokes an *avoidance* response. Further avoidance-attraction and flow-through activity testing on

Table 3.1 Summary of larval nucleobase compound avoidance-attraction testing.

Odourant class		**Nucleotides**							**Nucleosides**		**Nucleobases**		
Odourant		***ATP***	***AMP***	***GMP***	***CMP****	***UMP****	***IMP***	***NT mixture****	***Adenosine***	***Guanosine***	***Adenine***	***Hypoxanthine***	***H3NO***
Age	**5dpf**	attraction	-	attraction	-	-	-	-	avoidance	-	avoidance	-	-
	6dpf	-	-	-	-	-	attraction	-	-	-	-	-	-
	7dpf	avoidance	avoidance	-	-	-	-	-	-	-	-	avoidance	-

Data was analyzed by CM videogram analysis. Asterisks indicate pyrimidine containing compounds.

purine rings with and without the nitrogen-oxide group should be performed to test this new hypothesis.

Secondly, of the two nucleosides tested only adenosine showed avoidance while guanosine was behaviourally inactive. Between the two structures, the only difference is the purine ring; therefore the results suggest that the adenine ring is the active structural component, not the sugar. More nucleosides with diverse nucleobases should be tested to confirm this theory.

Finally, due to the feeding response seen in other species, attractive responses to nucleotides were hypothesized. Instead the findings were conflicting, and as described in the results, mixed avoidance and attraction was observed depending on the age and the odourant structure. The data is best discussed by breaking it down into simple observations.

Of the nucleotides tested only compounds with purine rings (ATP, AMP, GMP and IMP) elicited avoidance-attraction responses, while those containing pyrimidine rings (CMP and UMP) did not. This was unexpected as cytidine and uridine containing nucleotides effectively initiated gorging behaviour in the kissing bug and CTP stimulated spiny lobster chemosensory cells [64, 65, 81]. From these studies it was hypothesized that the phosphate groups in nucleotides would drive the behavioural responses. Conversely, it appears that for larval zebrafish, nucleobase structure is the determining factor. Interestingly, in other species (*Haliotis discus* and *Misgurnus anguillicaudatus*), neither CMP nor UMP increased feeding responses, however the pyrimidine thymidine was effective [39]. Consequently, additional pyrimidines, including thymidine, should be tested in zebrafish to determine whether pyrimidines can evoke avoidance-attraction behaviour. These odourants should also be tested in a flow through assay as they may be compounds that elicit a change in activity rather than an avoidance-attraction response.

Interestingly, the nucleotide mixture did not evoke any avoidance-attraction response despite containing behaviourally active AMP and GMP. This may be a result of the ratio and combination of the compounds within the mixture. However, nucleotides are most likely to appear in nature as mixtures therefore additional combinations should be tested that better represent nucleotide composition of food and conspecific tissue extracts.

Considering the responses observed by adenine and hypoxanthine (deaminated adenine), it follows that ATP, AMP and IMP (deaminated AMP) all also elicited avoidance-attraction behaviours. This again suggests the critical role of the nucleobase component in odourant reception and follows trends in previous studies that showed that adenine containing molecules evoked the most potent responses [64, 65]. It is also important to note that all compounds containing adenine had behavioural responses later in the trial, specifically during 5-7min and/or 7-10min. This further supports their potency in inducing avoidance-attraction behaviour as these time bins are larger than those that appear earlier in the trial. A response observed in the longer time bins would need to persist for the majority of the frames and therefore it is more likely that the persisting odourant effect is real rather than a random response.

What doesn't match previous trends, are the persistent potent avoidance-attraction responses to nucleobases with fewer phosphates. Although the degree of the response was not identified in fish studies, insect studies suggested decreased efficacy with decreased phosphates [64, 80, 81]. In the present study, there was no observed relationship between the number of phosphates and the capacity to evoke behaviours. Admittedly, these results do not include comparisons between odourants (e.g. ATP vs. AMP). Therefore to further confirm this observation, future studies should include dual odourant trials (one per side) to determine if compounds with additional phosphates are more effective than compounds with fewer phosphates.

The contradictory response to phosphates was also apparent in the strong avoidance responses to ATP and AMP. These findings were unexpected due to the correlations of these compounds to feeding events in other species [35, 39, 64, 80, 81]. Why might larval zebrafish avoid these stimuli? Perhaps it is an issue of age. Recalling the study examining food uptake of nucleotide enriched agar, the nucleotides actually deterred eating in Atlantic salmon parr but stimulated feeding in adult rainbow trout [35]. This begs the question, is there a reason for younger fish to be deterred by nucleotides? There are at least two potential explanations: 1) naivety to stimulus or 2) differing ecological significance. In the case of salmon parr, the fish have not yet made their oceanic migration and it is possible that the nucleotides from shrimp are not familiar as food odourant. In this respect, the fish are not responsive and wary of the chemical cue. The same is possible for larval zebrafish in this study as they have not yet been exposed to food and therefore may not associate nucleotides to feeding. As for ecological differences, considering that nucleotides are found in biological tissues such as blood and make up significant components of some invertebrate and fish extracts, ecologically, salmon parr and zebrafish larvae are more likely to be the prey to invertebrates and other fish as opposed to the predators [35, 80, 82, 87].

To further complicate an easy interpretation of my findings; the attractive response elicited by GMP contradicts the rationale suggested up until this point. Since guanosine evoked no response, but GMP did, this *does* suggest that the phosphate presence alters odourant efficacy. However because not all the tested nucleotides evoked a response (UMP and CMP did not), the presence of phosphate alone cannot be enough to activate the receptor and initiate the behavioural response. This leaves two possible answers 1) odourant receptor interaction is

instead based on the 3D structure and therefore is influenced by the collective form of the nucleotide as a whole, or 2) GMP is circumventing the odourant-receptor interaction.

Synthesizing my findings, the idea of the behavioural determinant being the 3D nucleotide (or nucleobase) structure as a whole is actually supported. From the odourants tested, purine ring structures similar to adenine were the ones that evoked responses. Deviations from adenine (changes in functional group and sugar addition) reduced efficacy, suggesting that perhaps there is an adenine specific receptor. Of the nucleotides, phosphate presence alone was not enough to induce avoidance-attraction behaviour. However, purine rings in combination with phosphate(s) did evoke changes in behaviour, while pyrimidine rings with phosphates did not (Figure 2.29). This suggests the presence of an additional receptor specific for the purine-phosphate interaction of the larger molecule or, perhaps phosphate addition allows for better purine-receptor interaction than with nucleosides. Additional nucleotides with diverse structures should be tested to confirm this theory.

As for bypassing the odourant receptor, notably, olfactory neurons do have cyclic nucleotide-gated (CNG) channels that open in response to elevated cyclic nucleotides (cAMP and cGMP) allowing for the influx of calcium and subsequent depolarization of the neuron [89, 90]. While larval zebrafish begin expressing CNG cation channels in the olfactory placode at ~24hours post fertilization and CNGs can be activated by cGMP, there is no evidence to suggest that GMP also acts as a secondary messenger or is modified to a cyclic form [31, 90]. The biochemical intricacies of odourant-receptor structure and GMP as a secondary messenger are beyond the scope of this thesis, but do merit further investigation.

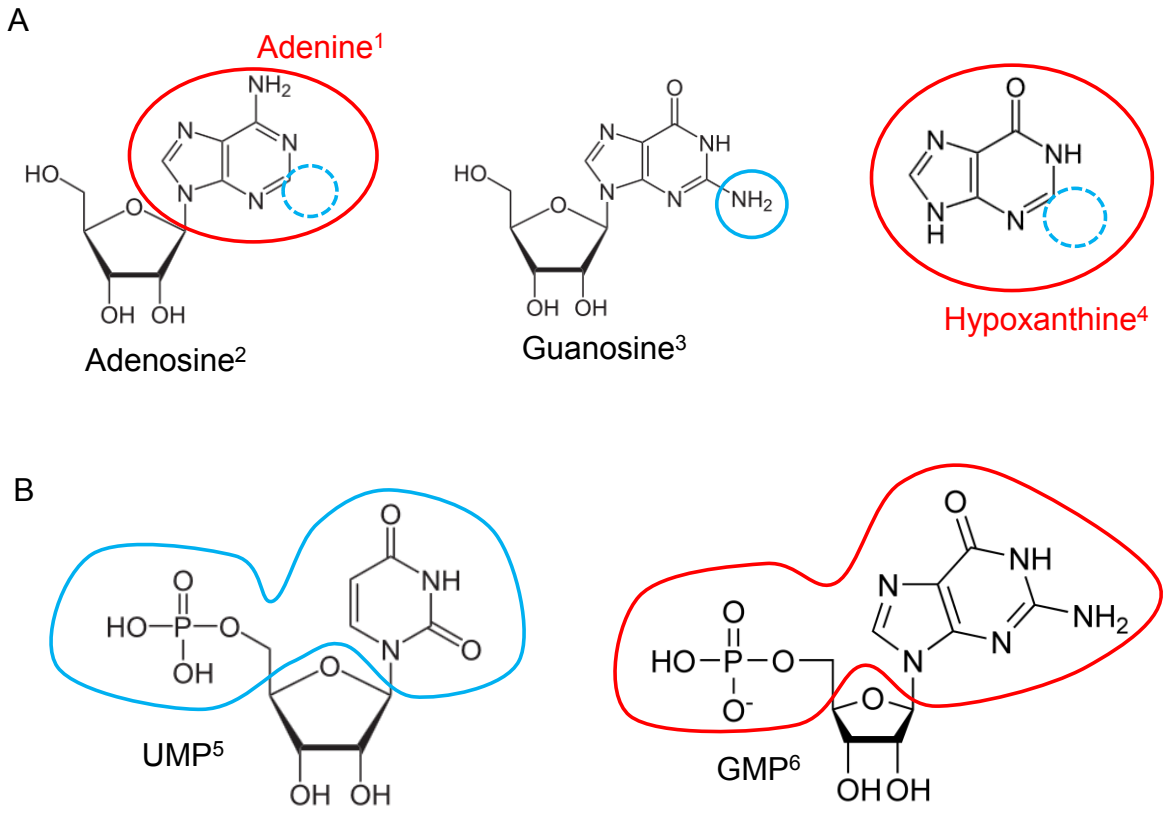


Figure 2.29. Purine ring functional groups and ring-phosphate interactions affect avoidance-attraction behaviour. Red and blue lines outline active and inactive structures respectively. Red text refers to chemical structures circled in red. Panel A) shows variation in purine function groups. Panel B) shows differences in ring-phosphate interactions.

[1] By Pepemonbu (Made with BkChem and Inkscape.) [GFDL (http://www.gnu.org/copyleft/fdl.html) or CC-BY-SA-3.0 (http://creativecommons.org/licenses/by-sa/3.0/)], via Wikimedia Commons

[2] "Adenosin" by NEUROtiker - Own work. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Adenosin.svg#/media/File:Adenosin.svg

[3] "Guanosin" by NEUROtiker - Own work. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Guanosin.svg#/media/File:Guanosin.svg

[4] "Hypoxanthin" by NEUROtiker - Own work. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Hypoxanthin.svg#/media/File:Hypoxanthin.svg

[5] "Uridinmonophosphat protoniert" by NEUROtiker - Own work. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Uridinmonophosphat_protoniert.svg#/media/File:Uridinmonophosphat_protoniert.svg

[6] "GMP chemical structure". Licensed under CC BY-SA 3.0 via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:GMP_chemical_structure.png#/media/File:GMP_chemical_structure.png

Olfactory-evoked behaviours do vary with age

Centre of mass data revealed that most odourant responses were observed at 5 and 7 dpf, while only IMP evoked a response (attraction) at 6 dpf. It is particularly interesting that there were distinctly fewer responses at 6 dpf. The fact that 6 dpf is the suspected olfactory imprinting window in zebrafish larvae is potentially an underlying reason for my findings. Previous studies have shown that zebrafish only develop the ability for kin odour recognition if exposed at 6 dpf [84, 85]. Furthermore, at 6 dpf larvae also exhibit an increase in thyroid hormone receptor β mRNA and elevated whole body thyroxine [91, 92]. In salmon (*Oncorhynchus kisutch*), a species known to imprint the odours of their natal stream, it has been shown that elevated plasma thyroxine is associated with olfactory epithelium proliferation [93]. The behavioural and hormonal evidence has produced this theory of a 6 dpf olfactory imprinting window. If true, it is possible that enhanced proliferation and dynamic status of olfactory tissue at this time impairs or confuses odourant interpretation. This theory, however, is relatively new and therefore olfactory histology at 6 vs. 7 dpf should be conducted to test this hypothesis. Nevertheless, in future it will likely be more beneficial to simply test larvae at 5 and 7 dpf.

An explanation for responses that are present at 7 dpf but not 5 dpf, for example 7 dpf AMP, may be asynchronous expression of receptors. Earlier studies have identified that the expression onset of odourant receptors varies in the age [30, 31]. It is therefore possible that receptors specific to AMP are not expressed until 7 dpf.

The diversity in responses observed at 5 vs.7 dpf does not match the expectation that larvae will be more responsive with increasing age. Neither were younger ages more prone to avoidance since attraction responses were seen at 5, but not 7 dpf. The absence in trend concerning

response type and prevalence in relation to fish age was ultimately unexpected and the rationale behind this finding remains unknown. Due to the stochastic nature of odourant receptor expression, it is possible that larvae have changing ratios of different receptor types at each age which modifies their response, but without molecular evidence this is purely speculation. Until the nucleotide associated odourant receptors are identified, the underlying mechanisms will remain unknown. In the short-term, older larvae (8-10 dpf) should be tested in order to better determine relationships between age and odourant response.

Repeated exposures diminish behavioural responses

Effective olfactory conditioning in zebrafish and the potential for naivety as factor influencing response, have led to the expectation that repeat trials without behavioural consequence would increase odourant familiarity and therefore decrease avoidance responses [37]. Unfortunately, the right side bias within the repeated 5-7 dpf exposures largely occluded the interpretation of the results, especially in terms of EM and FE. Those experiments will need to be conducted again for verification of the results. Nevertheless, there was an observed reduction in avoidance of AMP in 7 dpf fish that were repeatedly exposed vs. those that were naïve. This result further stresses the need for continued investigation since larval fish could be responding with avoidance due to naivety, not due to innate fear. Repeat testing with additional avoidance stimulating odourants and older zebrafish should be conducted to further this hypothesis. However, a notable limitation in repeat trials is the reduced survival with experiment progression (data not shown). This is likely the result of increased handling and elevated stress, therefore in future it may be beneficial to increase the number of rest days between trials.

Future directions

Apart from increasing the number of trials, odourants, concentrations and expanding the ages tested, there are several other avenues to explore in building on the newfound results concerning zebrafish nucleobase-evoked behaviour.

The question remains as to whether or not the zebrafish tested are in fact detecting the nucleobase compounds via the olfactory system. From previous electrophysiological studies in larval zebrafish and adult species of other fish, it is known that the olfactory system can be stimulated by nucleotides [10, 11]. However there is no paired study that confirms or isolates olfaction as the affected mechanism in this thesis.

The zebrafish could in fact be detecting the nucleobase compounds via gustatory, or taste, receptors instead. An electrophysiology study identified positive gustatory neural stimulation in Atlantic halibut to AMP, IMP and GMP, although it should be noted that responses were at much higher concentrations than tested in my experiments ($1x10^{-3}$ and $1x10^{-2}$M vs. $1x10^{-5}$M) [79]. As functional taste receptors develop at 4-5 dpf in zebrafish larvae, it is possible that larvae were detecting compounds via gustation [94]. However, since some G-proteins such as G_{ib} are expressed in both olfactory and gustatory tissue in zebrafish, there is no reason to conclude that nucleobase detection must be exclusive to one sensory system [26]. Zebrafish may detect this chemical class via both taste and smell. Importantly, this would not negate my findings; it would simply clarify the mechanism. Determination of which chemosensory system is involved could be theoretically deduced through identifying recently active neurons via *c-fos* expression. *C-fos* is an immediate early gene whose transcription has been previously used as a marker of neural activity in zebrafish in both olfactory specific and non-specific tissues [47, 95-97]. Odourant exposures and subsequent *c-fos* labelling via in-situ hybridization and immunohistochemistry

were attempted in this study, however experiments were unsuccessful (data not shown). Extensive variations in exposure and detection methods were explored, but for undetermined reasons results remained inconclusive. An alternative method known as CaMPARI (calcium-modulated photoactivatable ratiometric integrator) may offer a potential solution. Zebrafish larvae expressing CaMPARI that undergo the same behavioural assay during exposure to light, will exhibit permanent fluorescent staining in calcium depolarized neurons [98]. In this way, the sensory system responsible for the behavioural response could be identified.

As we delve deeper into the mechanism of detection, the identity of nucleobase receptors and secondary messenger systems is one that is particularly fascinating. A variety of purinergic receptors that may bind to the nucleobase compounds tested here have been identified in goldfish, lobster, and mouse chemosensory tissues [65, 99, 100]. To identify possible receptors, larval zebrafish were raised in nucleobase compound (adenine family) enriched EM until 7 dpf. The exposure period encompasses the predicted imprinting window in larvae (6 dpf), therefore it expected that the relevant nucleobase olfactory receptors will have heightened transcription compared to unexposed larvae [85]. Future transcriptome analyses of these exposed vs. unexposed fish will provide clues as to the putative receptors and mechanisms of nucleobase detection. Knowing the receptors involved will be paramount to understanding the variation in behavioural efficacy of nucleobase compounds.

Conclusion

Overall, this study effectively identified behaviourally active nucleobase compounds during early larval development. The data presented in regards to nucleobase responses has the high variability that is normally attributed to behavioural studies and has been especially noted in regards to larval zebrafish[50]. In fact, behavioural variation is so common that it has led to the

well-known Harvard Law of Animal Behaviour: "Under carefully controlled experimental circumstances, an animal will behave as it damned well pleases." This comical yet truthful phenomenon of behavioural variation is often countered by having large sample sizes in order to find significant responses. The data that I have presented in this chapter does have variation, and the differing responses between the ages tested may seem random. However, behavioural data rarely exhibits normality and statistical significance with sample sizes of 8-10. The fact that these experiments yielded significant results combats the argument of randomness and is a testament to both the validity of the methods applied and the potency of the odourants tested. Although the results described here are largely yes-no answers to the question of responsiveness and they provide only hints as to the positive or negative associations of each compound, the observations shed light on the diversity of responses between fish species and life stages. Further investigation will be crucial in understanding which nucleobase structures are involved in survival associated behaviours. As researchers strive to comprehend the intricacies of the olfactory system in fish, science moves towards tremendous potential for its application. By endeavouring to understand olfaction in terms of the structural efficacy of odourants, potential disruptors of this essential sensory system can be identified. Researchers can now move on to determine whether environmental contaminants with similar structures to the identified odourants, mimic or prevent the naturally induced behaviour. It is only through understanding how fish survive that we can prevent their endangerment.

Chapter 4: General conclusion

The overarching objective of this thesis was to further characterize olfactory-evoked behaviour in larval zebrafish. Throughout the investigation, the research focused on the poorly understood nucleobase compounds as the odourant class of interest. However, to identify behaviours elicited by novel odourants, both effective methods and positive controls for testing larvae were required. This thesis described the successful creation and validation of two apparatuses designed for the purpose of odourant testing in young fish.

The flow-through system is greatly improved upon from pre-existing experimental setups because as a temperature regulated, consistent flow environment, it limits behavioural stimuli to odourant exposures alone. The system is able to test multiple subjects simultaneously and several odourants per subject. Through validation studies, it was determined that the synthesized odourant H3NO effectively modified behaviour in 7 dpf larvae. This was the earliest identified response to H3NO and as far as I know the first characterized 'alarm' activity in larvae. The potential applications for this method in further describing odourant, or toxicant and pharmaceutical, evoked behaviour are indeed promising.

The second apparatus, the avoidance-attraction trough is the embodiment of a well-studied concept applied to a new and simple small-scale device. The method effectively showed larval avoidance of EE and in doing so identified another new positive control. Validation experiments also showed that fish had no innate attraction to food extract, which raised several interesting research questions regarding innate vs. learned responses.

Effective development of the avoidance-attraction trough enabled investigation of larval avoidance-attraction responses to nucleobase compounds. It was confirmed that nucleobases, nucleosides and nucleotides of differing phosphates are all capable of inducing avoidance-attraction responses in zebrafish larvae. Although further testing is required, existing evidence suggests that the nucleobase structure governs behavioural responses. Responses also varied with larval age, which merits further study at the receptor level for changes during early development.

Reflecting on the entirety of the work is it evident now more than ever that the use of multiple behavioural metrics and equipment optimized for each experiment is crucial to odourant characterization. For example, H3NO, an odourant that elicited a change in activity, did not evoke an aversive response. As discussed in Chapters 2 and 3, classical alarm responses such as the increased erratic movements captured in the flow through system, cannot be observed in the avoidance-attraction trough. Likewise, foraging behaviour for which there were observed trends in the flow-through system cannot be determined from the avoidance attraction assay. These findings are supported by another study which showed that some odourants can elicit either an avoidance-attraction response or a change in activity, but not both[39]. Taking this into consideration, while some nucleobase compounds tested here can now be characterized as ineffective attractants or deterrents, further testing may show that these compounds evoke other behavioural responses.

While many compounds have been shown to stimulate fish olfactory tissue, ultimately, a chemical should only be classified as an odourant if it has downstream physiological or behavioural effects. Behavioural studies like those presented within this thesis serve to elucidate the natural significance of odourants, and also provide clues as to structural relationships between odourants and receptors. The small field of fish olfaction leaves much to be discovered,

and true to the nature of basic science, the results of these experiments have led more to new research questions than to concrete answers. Nevertheless, the validated methods and positive controls described here have established the foundation for future investigators.

Bibliography

1. Derby, C.D. and P.W. Sorensen, *Neural processing, perception, and behavioral responses to natural chemical stimuli by fish and crustaceans.* Journal of Chemical Ecology, 2008. **34**(7): p. 898-914.
2. Tierney, K.B., et al., *Olfactory toxicity in fishes.* Aquatic Toxicology, 2010. **96**(1): p. 2-26.
3. Dew, W.A., A. Azizishirazi, and G.G. Pyle, *Contaminant-specific targeting of olfactory sensory neuron classes: Connecting neuron class impairment with behavioural deficits.* Chemosphere, 2014. **112**: p. 519-525.
4. Hansen, A. and Z. Eckart, *The Peripheral Olfactory Organ of the Zebrafish, Danio rerio: an Ultrastructural Study.* Chemical Senses, 1998. **23**(1): p. 39-48.
5. Hansen, A. and B. Zielinski, *Diversity in the olfactory epithelium of bony fishes: Development, lamellar arrangement, sensory neuron cell types and transduction components.* Journal of Neurocytology, 2005. **34**(3-5): p. 183-208.
6. Hara, T.J., *Olfaction in fish.* Progress in Neurobiology (Oxford), 1975. **5**(4): p. 271-335.
7. Hara, T.J., *Olfaction and gustation in fish: an overview.* Acta Physiologica Scandinavica, 1994. **152**(2): p. 207-217.
8. Michel, W.C., et al., *Evidence of a novel transduction pathway mediating detection of polyamines by the zebrafish olfactory system.* Journal of Experimental Biology, 2003. **206**(10): p. 1697-1706.
9. Rolen, S.H., et al., *Polyamines as olfactory stimuli in the goldfish Carassius auratus.* Journal of Experimental Biology, 2003. **206**(10): p. 1683-1696.

10. Nikonov, A.A. and J. Caprio, *Electrophysiological evidence for a chemotopy of biologically relevant odors in the olfactory bulb of the channel catfish.* Journal of Neurophysiology, 2001. **86**(4): p. 1869-1876.

11. Li, J., et al., *Early Development of Functional Spatial Maps in the Zebrafish Olfactory Bulb.* The Journal of Neuroscience, 2005. **25**(24): p. 5784-5795.

12. Sorensen, P.W., et al., *Sulfated 17,20-beta-dihydroxy-4-pregnen-3-one functions as a potent and specific olfactory stimulant with pheromonal actions in the goldfish.* General and Comparative Endocrinology, 1995. **100**(1): p. 128-142.

13. Laberge, F. and T.J. Hara, *Behavioural and electrophysiological responses to F-prostaglandins, putative spawning pheromones, in three salmonid fishes.* Journal of Fish Biology, 2003. **62**(1): p. 206-221.

14. Belanger, R.M., M.D. Pachkowski, and N.E. Stacey, *Methyltestosterone-Induced Changes in Electro-olfactogram Responses and Courtship Behaviors of Cyprinids.* Chemical Senses, 2010. **35**(1): p. 65-74.

15. Yacoob, S.Y., H.I. Browman, and P.A. Jensen, *Electroencephalogram recordings from the olfactory bulb of juvenile (0 year) Atlantic cod in response to amino acids.* Journal of Fish Biology, 2004. **65**(6): p. 1657-1664.

16. Hansen, A., et al., *Correlation between olfactory receptor cell type and function in the channel catfish.* Journal of Neuroscience, 2003. **23**(28): p. 9328-9339.

17. Yoshihara, Y., *Molecular Genetic Dissection of the Zebrafish Olfactory System*, in *Chemosensory Systems in Mammals, Fishes, and Insects*, S. Korsching and W. Meyerhof, Editors. 2009, Springer Berlin Heidelberg. p. 1-19.

18. Vielma, A., et al., *The elusive crypt olfactory receptor neuron: evidence for its stimulation by amino acids and cAMP pathway agonists.* Journal of Experimental Biology, 2008. **211**(15): p. 2417-2422.

19. Bazaes, A. and O. Schmachtenberg, *Odorant tuning of olfactory crypt cells from juvenile and adult rainbow trout.* Journal of Experimental Biology, 2012. **215**(10): p. 1740-1748.

20. Sato, Y., N. Miyasaka, and Y. Yoshihara, *Mutually Exclusive Glomerular Innervation by Two Distinct Types of Olfactory Sensory Neurons Revealed in Transgenic Zebrafish.* The Journal of Neuroscience, 2005. **25**(20): p. 4889-4897.

21. Morita, Y. and T.E. Finger, *Differential projections of ciliated and microvillous olfactory receptor cells in the catfish, Ictalurus punctatus.* The Journal of Comparative Neurology, 1998. **398**(4): p. 539-550.

22. Alioto, T.S. and J. Ngai, *The odorant receptor repertoire of teleost fish.* Bmc Genomics, 2005. **6**.

23. Hashiguchi, Y., Y. Furuta, and M. Nishida, *Evolutionary Patterns and Selective Pressures of Odorant/Pheromone Receptor Gene Families in Teleost Fishes.* Plos One, 2008. **3**(12): p. e4083.

24. Amoore, J.E., *Current status of steric theory of odor.* Annals of the New York Academy of Sciences, 1964. **116**(A2): p. 457.

25. Brookes, J.C., A.P. Horsfield, and A.M. Stoneham, *The swipe card model of odorant recognition.* Sensors (Basel, Switzerland), 2012. **12**(11): p. 15709-15749.

26. Oka, Y. and S.I. Korsching, *Shared and Unique G Alpha Proteins in the Zebrafish Versus Mammalian Senses of Taste and Smell.* Chemical Senses, 2011. **36**(4): p. 357-365.

27. Hansen, A., et al., *Olfactory receptor neurons in fish: Structural, molecular and functional correlates.* Chemical Senses, 2005. **30**: p. I311-I311.

28. Vitebsky, A., et al., *Isolation and characterization of the laure olfactory behavioral mutant in the zebrafish, Danio rerio.* Developmental Dynamics, 2005. **234**(1): p. 229-242.

29. Lindsay, S.M. and R.G. Vogt, *Behavioral Responses of Newly Hatched Zebrafish (Danio rerio) to Amino Acid Chemostimulants.* Chemical Senses, 2004. **29**(2): p. 93-100.

30. Argo, S., F. Weth, and S.I. Korsching, *Analysis of penetrance and expressivity during ontogenesis supports a stochastic choice of zebrafish odorant receptors from predetermined groups of receptor genes.* European Journal of Neuroscience, 2003. **17**(4): p. 833-843.

31. Barth, A.L., N.J. Justice, and J. Ngai, *Asynchronous Onset of Odorant Receptor Expression in the Developing Zebrafish Olfactory System.* Neuron, 1996. **16**(1): p. 23-34.

32. Colwill, R.M. and R. Creton, *Locomotor behaviors in zebrafish (Danio rerio) larvae.* Behavioural Processes, 2011. **86**(2): p. 222-229.

33. Kimmel, C.B., et al., *Stages of embryonic development of the zebrafish.* Developmental Dynamics, 1995. **203**(3): p. 253-310.

34. Jones, K.A. and T.J. Hara, *Behavioural responses of fishes to chemical cues: results from a new bioassay.* Journal of Fish Biology, 1985. **27**(4): p. 495-504.

35. Mearns, K.J., et al., *Feeding behaviour in adult rainbow trout and Atlantic salmon parr, elicited by chemical fractions and mixtures of compounds identified in shrimp extract.* Aquaculture, 1987. **64**(1): p. 47-63.

36. Bhinder, G. and K. Tierney, *Olfactory-Evoked Activity Assay for Larval Zebrafish*, in *Zebrafish Protocols for Neurobehavioral Research*, A.V. Kalueff and A.M. Stewart, Editors. 2012, Humana Press. p. 71-84.

37. Braubach, O.R., et al., *Olfactory conditioning in the zebrafish (Danio rerio).* Behavioural Brain Research, 2009. **198**(1): p. 190-198.

38. Steele, C.W., D.W. Owens, and A.D. Scarfe, *Attraction of zebrafish, Brachydanio rerio, to alanine and its suppression by copper.* Journal of Fish Biology, 1990. **36**(3): p. 341-352.

39. Miyasaki, T. and K. Harada, *Effects of specific purine and pyrimidine compounds on the ingestion of test diets by the abalone Haliotis discus and the oriental weatherfish Misgurnus anguillicaudatus.* Marine and Freshwater Research, 2003. **54**(3): p. 235-241.

40. Jesuthasan, S.J. and A.S. Mathuru, *The Alarm Response in Zebrafish: Innate Fear in a Vertebrate Genetic Model.* Journal of Neurogenetics, 2008. **22**(3): p. 211-228.

41. Brown, G., et al., *Ostariophysan Alarm Pheromones: Laboratory and Field Tests of the Functional Significance of Nitrogen Oxides.* Journal of Chemical Ecology, 2000. **26**(1): p. 139-154.

42. Mathuru, Ajay S., et al., *Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish.* Current biology : CB, 2012. **22**(6): p. 538-544.

43. Brown, G.E., J.C. Adrian, and M.L. Shih, *Behavioural responses of fathead minnows to hypoxanthine-3-N-oxide at varying concentrations.* Journal of Fish Biology, 2001. **58**(5): p. 1465-1470.

44. Speedie, N. and R. Gerlai, *Alarm substance induced behavioral responses in zebrafish (Danio rerio).* Behavioural Brain Research, 2008. **188**(1): p. 168-177.

45. Parra, K.V., J.C. Adrian, and R. Gerlai, *The synthetic substance hypoxanthine 3-N-oxide elicits alarm reactions in zebrafish (Danio rerio).* Behavioural Brain Research, 2009. **205**(2): p. 336-341.

46. Oliveira, T.A., et al., *Death-associated odors induce stress in zebrafish.* Hormones and Behavior, 2014. **65**(4): p. 340-344.

47. Hussain, A., et al., *High-affinity olfactory receptor for the death-associated odor cadaverine.* Proceedings of the National Academy of Sciences, 2013. **110**(48): p. 19579-19584.

48. Idler, D.R., U.H.M. Fagerlund, and H. Mayoh, *Olfactory perception in migrating salmon .1. L-serine, a salmon repellent in mammalian skin.* Journal of General Physiology, 1956. **39**(6): p. 889-892.

49. Krishnan, S., et al., *The Right Dorsal Habenula Limits Attraction to an Odor in Zebrafish.* Current Biology, 2014. **24**(11): p. 1167-1175.

50. Shamchuk, A.L. and K.B. Tierney, *Phenotyping stimulus evoked responses in larval zebrafish.* Behaviour, 2012. **149**(10-12): p. 1177-1203.

51. Steele, C.W., A.D. Scarfe, and D.W. Owens, *Effects of group size on the responsiveness of zebrafish, Brachydanio rerio (Hamilton Buchanan), to alanine, a chemical attractant.* Journal of Fish Biology, 1991. **38**(4): p. 553-564.

52. Steenbergen, P.J., M.K. Richardson, and D.L. Champagne, *Patterns of avoidance behaviours in the light/dark preference test in young juvenile zebrafish: A pharmacological study.* Behavioural Brain Research, 2011. **222**(1): p. 15-25.

53. Engeszer, R.E., et al., *Timing and plasticity of shoaling behaviour in the zebrafish, Danio rerio.* Animal Behaviour, 2007. **74**(5): p. 1269-1275.

54. Stewart, W.J., G.S. Cardenas, and M.J. McHenry, *Zebrafish larvae evade predators by sensing water flow.* Journal of Experimental Biology, 2013. **216**(3): p. 388-398.

55. Müller, U.K. and J.L. van Leeuwen, *Swimming of larval zebrafish: ontogeny of body waves and implications for locomotory development.* Journal of Experimental Biology, 2004. **207**(5): p. 853-868.

56. Dempsey, C.H., *Chemical Stimuli as a factor in feeding and intraspecific behaviour of Herring Larvae.* Journal of the Marine Biological Association of the United Kingdom, 1978. **58**(03): p. 739-747.

57. Richendrfer, H., et al., *On the edge: Pharmacological evidence for anxiety-related behavior in zebrafish larvae.* Behavioural Brain Research, 2012. **228**(1): p. 99-106.

58. Schnörr, S.J., et al., *Measuring thigmotaxis in larval zebrafish.* Behavioural Brain Research, 2012. **228**(2): p. 367-374.

59. Kalueff, A.V., et al., *Towards a Comprehensive Catalog of Zebrafish Behavior 1.0 and Beyond.* Zebrafish, 2013. **10**(1): p. 70-86.

60. Chen, T.-H., C.-C. Lin, and P.-J. Meng, *Zinc oxide nanoparticles alter hatching and larval locomotor activity in zebrafish (Danio rerio).* Journal of Hazardous Materials, 2014. **277**(0): p. 134-140.

61. Pfeiffer, W., *The Distribution of Fright Reaction and Alarm Substance Cells in Fishes.* Copeia, 1977. **1977**(4): p. 653-665.

62. Muto, A. and K. Kawakami, *Prey capture in zebrafish larvae serves as a model to study cognitive functions.* Frontiers in Neural Circuits, 2013. **7**: p. 110.

63. Brown, G., et al., *Nitrogen Oxides Elicit Antipredator Responses in Juvenile Channel Catfish, But Not in Convict Cichlids or Rainbow Trout: Conservation of the Ostariophysan Alarm Pheromone.* Journal of Chemical Ecology, 2003. **29**(8): p. 1781-1796.

64. Friend, W.G. and J.J.B. Smith, *Feeding in Rhodnius prolixus: Potencies of nucleoside phosphates in initiating gorging.* Journal of Insect Physiology, 1971. **17**(7): p. 1315-1320.

65. Carr, W.E.S., et al., *Olfactory receptors of the spiny lobster: ATP-sensitive cells with similarities to P_2-type purinoceptors of vertebrates.* Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology, 1986. **158**(3): p. 331-338.

66. Westerfield, M., *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Brachydanio Rerio)*. 1993: M. Westerfield.

67. MacPhail, R.C., et al., *Locomotion in larval zebrafish: Influence of time of day, lighting and ethanol.* NeuroToxicology, 2009. **30**(1): p. 52-58.

68. Wilson, J.M., R.M. Bunte, and A.J. Carty, *Evaluation of Rapid Cooling and Tricaine Methanesulfonate (MS222) as Methods of Euthanasia in Zebrafish (Danio rerio).* Journal of the American Association for Laboratory Animal Science : JAALAS, 2009. **48**(6): p. 785-789.

69. Zar, J.H., *Biostatistical Analysis*. 4th ed. 1999, Upper Saddle River, New Jersey: Prentice Hall, Inc.

70. Kawashima, H. and I. Kumashiro, *Studies of Purine N-Oxides. III. The Synthesis of Purine 3-N-Oxides.* Bulletin of the Chemical Society of Japan, 1969. **42**(3): p. 750-755.

71. Brown, G.E., et al., *The effects of reduced pH on chemical alarm signalling in ostariophysan fishes.* Canadian Journal of Fisheries & Aquatic Sciences, 2002. **59**(8): p. 1331.

72. Conover, W.J., *Practical Nonparametric Statistics*. 2nd ed. 1980, New York: John Wiley & Sons, Inc.

73. Tierney, K.B., et al., *Relating olfactory neurotoxicity to altered olfactory-mediated behaviors in rainbow trout exposed to three currently-used pesticides.* Aquatic Toxicology, 2007. **81**(1): p. 55-64.

74. Pelkowski, S.D., et al., *A novel high-throughput imaging system for automated analyses of avoidance behavior in zebrafish larvae.* Behavioural Brain Research, 2011. **223**(1): p. 135-144.

75. de Esch, C., et al., *Locomotor activity assay in zebrafish larvae: Influence of age, strain and ethanol.* Neurotoxicology and Teratology, 2012. **34**(4): p. 425-433.

76. Li, Q., et al., *Differential behavioral responses of zebrafish larvae to yohimbine treatment.* Psychopharmacology, 2015. **232**(1): p. 197-208.

77. Ali, S., D.L. Champagne, and M.K. Richardson, *Behavioral profiling of zebrafish embryos exposed to a panel of 60 water-soluble compounds.* Behavioural Brain Research, 2012. **228**(2): p. 272-283.

78. Naoteru, K., et al., *Establishment of a novel experimental protocol for drug-induced seizure liability screening based on a locomotor activity assay in zebrafish.* Journal of Toxicological Sciences, 2014. **39**(4): p. 579-600.

79. Yacoob, S.Y. and H.I. Browman, *Olfactory and gustatory sensitivity to some feed-related chemicals in the Atlantic halibut (Hippoglossus hippoglossus).* Aquaculture, 2007. **263**(1–4): p. 303-309.

80. Romero, A. and C. Schal, *Blood constituents as phagostimulants for the bed bug Cimex lectularius L.* The Journal of Experimental Biology, 2014. **217**(4): p. 552-557.

81. Smith, J.J.B. and W.G. Friend, *Further studies on potencies of nucleotides as gorging stimuli during feeding in Rhodnius prolixus.* Journal of Insect Physiology, 1976. **22**(4): p. 607-611.

82. Carr, W.E.S., et al., *Stimulants of feeding behavior in fish: Analyses of tissues of diverse marine organisms.* Biological Bulletin, 1996. **190**(2): p. 149-160.

83. Byrd, C.A., et al., *Ontogeny of odorant receptor gene expression in zebrafish, Danio rerio.* Journal of Neurobiology, 1996. **29**(4): p. 445-458.

84. Hinz, C., et al., *Kin recognition in zebrafish, Danio rerio, is based on imprinting on olfactory and visual stimuli.* Animal Behaviour, 2013. **85**(5): p. 925-930.

85. Gerlach, G., et al., *Kin recognition in zebrafish: a 24-hour window for olfactory imprinting.* Proceedings of the Royal Society B-Biological Sciences, 2008. **275**(1647): p. 2165-2170.

86. Wyeth, R.C., et al., *Videograms: A Method for Repeatable Unbiased Quantitative Behavioral Analysis Without Scoring or Tracking*, in *Zebrafish Neurobehavioral Protocols*, A.V. Kalueff and J.M. Cachat, Editors. 2011. p. 15-33.

87. Engeszer, R.E., et al., *Zebrafish in the Wild: A Review of Natural History and New Notes from the Field.* Zebrafish, 2007. **4**(1): p. 21-U126.

88. Cachat, J., et al., *Measuring behavioral and endocrine responses to novelty stress in adult zebrafish.* Nat. Protocols, 2010. **5**(11): p. 1786-1799.

89. Zheng, J. and W.N. Zagotta, *Stoichiometry and Assembly of Olfactory Cyclic Nucleotide-Gated Channels.* Neuron, 2004. **42**(3): p. 411-421.

90. Goulding, E.H., et al., *Molecular cloning and single-channel properties of the cyclic nucleotide-gated channel from catfish olfactory neurons.* Neuron, 1992. **8**(1): p. 45-58.

91. Chang, J., et al., *Changes in Thyroid Hormone Levels during Zebrafish Development.* Zoological Science, 2012. **29**(3): p. 181-184.

92. Liu, Y.W. and W.K. Chan, *Thyroid hormones are important for embryonic to larval transitory phase in zebrafish.* Differentiation, 2002. **70**(1): p. 36-45.

93. Lema, S.C. and G.A. Nevitt, *Evidence that thyroid hormone induces olfactory cellular proliferation in salmon during a sensitive period for imprinting.* Journal of Experimental Biology, 2004. **207**(19): p. 3317-3327.

94. Hansen, A., K. Reutter, and E. Zeiske, *Taste bud development in the zebrafish, Danio rerio.* Developmental Dynamics, 2002. **223**(4): p. 483-496.

95. Kishimoto, N., et al., *Interhemispheric asymmetry of olfactory input-dependent neuronal specification in the adult brain.* Nat Neurosci, 2013. **16**(7): p. 884-888.

96. Baraban, S.C., et al., *Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression.* Neuroscience, 2005. **131**(3): p. 759-768.

97. deCarvalho, T.N., et al., *Aversive cues fail to activate fos expression in the asymmetric olfactory-habenula pathway of zebrafish.* Frontiers in Neural Circuits, 2013. **7**: p. 98.

98. Fosque, B.F., et al., *Labeling of active neural circuits in vivo with designed calcium integrators.* Science, 2015. **347**(6223): p. 755-760.

99. Vick, J.S. and R.J. Delay, *ATP excites mouse vomeronasal sensory neurons through activation of P2X receptors.* Neuroscience, 2012. **220**(0): p. 341-350.

100. Kolmakov, N.N., et al., *Analysis of the goldfish Carassius auratus olfactory epithelium transcriptome reveals the presence of numerous non-olfactory GPCR and putative receptors for progestin pheromones.* Bmc Genomics, 2008. **9**: p. 429-429.

Appendix A

Flow-through system troubleshooting and operation notes

There remain several issues with the operation of the flow-through system, primarily its tendency to breakdown, which is largely attributed to its components and its amateur fabrication. Additionally, all current components aside from the testing chambers are composed of plastic. For future applications involving the behaviour responses to toxicants, applicable materials should be switched to glass so that the system can be effectively cleaned between trials. For optimum functionality, the system should be reconstructed using perfusion system technology from AutoMate Scientific Inc. Currently, this equipment exceeds budget constraints; therefore until the system can be rebuilt there are several operating notes to consider.

First, it is important to be aware of the length of time required to set up and perform a flow-through trial and it should be noted that as operation time increases, as does the susceptibility to common malfunctions. Calibration of chamber flow through takes a minimum of 45 minutes, and if repairs are needed the time required has historically extended past 2 hours. Notably, post calibration an entire trial takes 127 minutes. Due to the time sensitive nature of behavioural experiments, trials must be in operation by 2:30pm daily or else the larvae are considered to be past the designated testing window. Theoretically, two experiments can be performed daily, however due to frequency of system malfunctions this has yet to be achieved. To preserve resources, odourant solutions should only be prepared immediately prior to trials and daily testing populations should be restricted to small numbers so animals are not wasted. Currently, chambers are designed to hold a single fish therefore a maximum of four fish are used per trial. Fish can be lost to the system during trial setup therefore it is recommended that at least ten fish are kept in reserve.

Second, air bubbles within the system cause two main issues with the trial including reduced flow rate into the funnel system and air flow into the odourant delivery chambers. When flow rate into the funnel system is reduced, the peristaltic pump overwhelms the flow rate and air instead of odourant solution is consequently pumped into the glass chambers. Air flow into the chambers results creates a bubble which disrupts the trial in three ways: it obstructs the camera view of the fish, it changes the fluid volume within the chamber and its entry acts as a secondary stimulus thereby altering fish behaviour. It is essential that air bubbles are avoided at all times. This is achieved in two different ways. The first way is the presence of drip chambers analogous to those found in intravenous fluid delivery systems. For optimum functionality, fluid level within the drips must be maintained. Therefore, prior to each trial to correct for any accumulation of air, drip chambers should be opened and filled three quarters full with EM. Odourant specific drip chambers should be filled similarly with the appropriate test solution. Drip chambers following the peristaltic pump should always be propped in an upright position to ensure appropriate fluid level. The second method to prevent air accumulation is priming and flushing delivery lines prior to trials. Using a 60ml EM filled syringe attached to the odourant delivery stopcocks, delivery lines can be primed and flushed to ensure prompt delivery and consistent flow rate respectively.

Third, fish loss during trials is another major impediment to experiment progress. The silver wires inserted in the narrowed portion of the glass chamber arms prevent the majority of larvae from entering inflow or outflow areas. However, fish that do enter these areas can be either injured or lost through the outflow. As it stands, robust 7 dpf larvae are sizable enough that fish loss is reduced, however the issue does persist. Insertion of a fluid permeable mesh within the

chamber arms is the suggested modification; however it should be noted this will require further prototyping and additional dye trials to re-evaluate the fluid dynamics.

Finally, the Masterflex tubing used for the peristaltic pump presents the greatest challenge. The tubing wears significantly during trials and needs to be regularly inspected and replaced. Inattention to tubing condition eventually leads to the tubing splitting during trials. This type of malfunction cannot be remedied in enough time to rescue the trial. Re-calibration post tubing replacement generally requires at least one hour. The Masterflex tubing can also develop a tendency to move through the pump i.e. be pumped through the cartridge. This can be prevented by fixing a butterfly clip on the Masterflex tubing directly prior to its entrance into the pump cartridge. The clip prevents the tubing from moving any further.

Appendix B

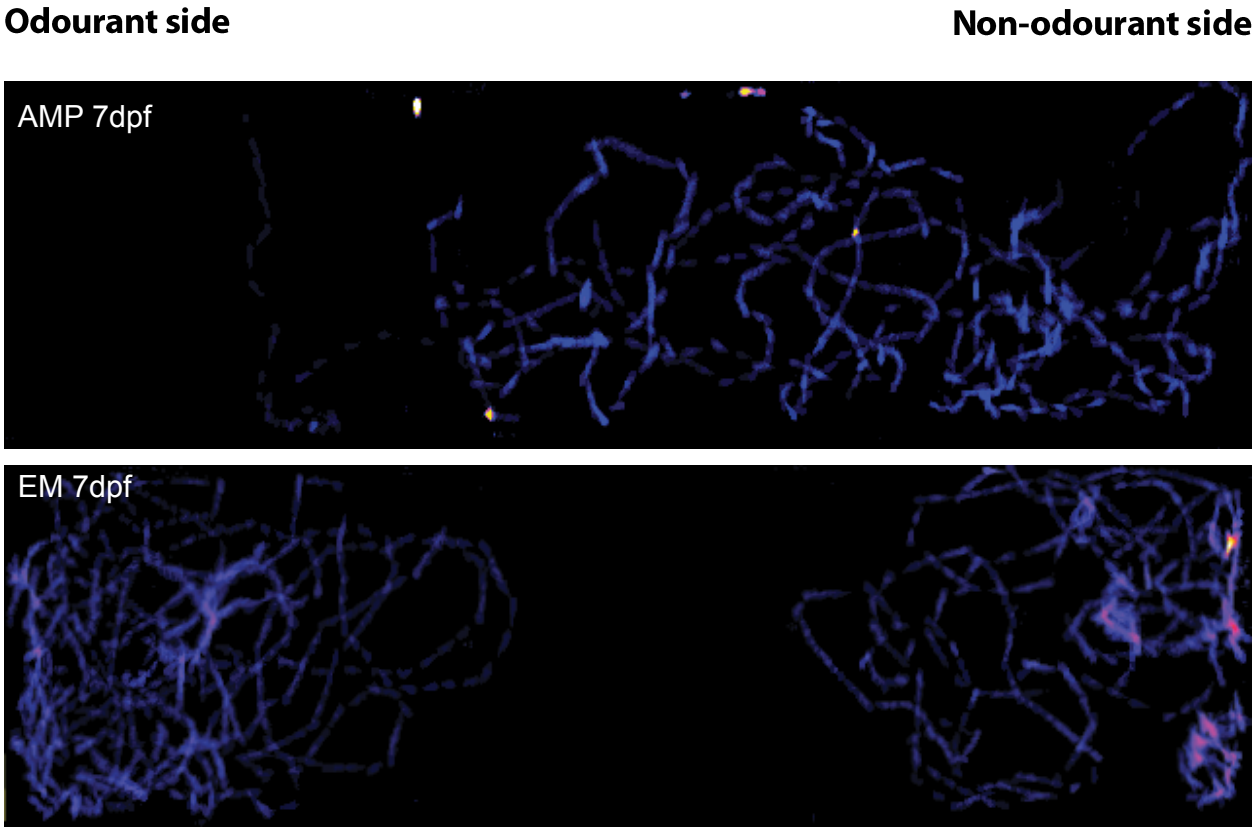
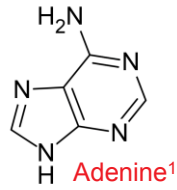


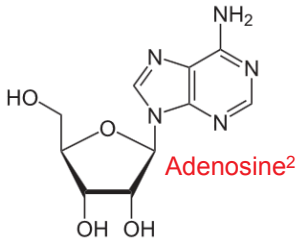
Figure B. Sample 7 dpf AMP vs. EM videograms. Images represent 7-10 min in single trials. Image contrast has been modified to enhance appearance. Note contrast is not altered during videogram analysis.

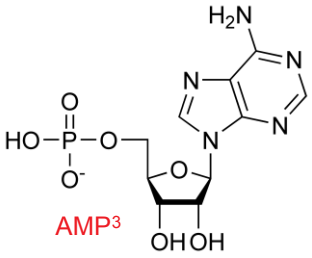
A detailed protocol outlining videogram creation can be found in Wyeth (2011)[86]. Summary images (ex. Figure B) are formed by compiling frames from a designated period of time in ImageJ. Essentially, the non-black areas indicate where fish were present during the time period and they hold numerical value. The centre of mass can be measured on the x-axis and it is influenced by where the bulk of the activity takes place (left-right). In control trials, when fish use the left and right sides evenly, the centre of mass is near the physical centre of the apparatus. In effective odourant trials, where the fish avoid or are attracted to the odourant side, the centre of mass is shifted away from the physical centre, away or towards the odourant respectively. Shifts in centre of mass from the absolute centre are compared between control and odourant trials to determine odourant efficacy in evoking avoidance-attraction behaviour.

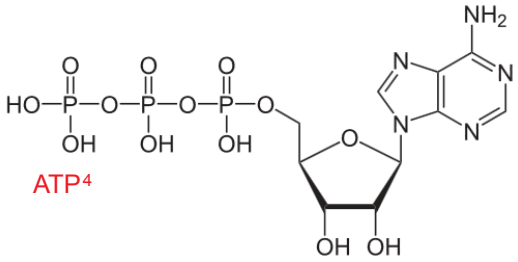
Appendix C

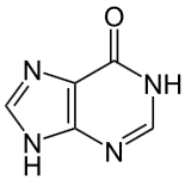
Avoidance-attraction inducing compounds:

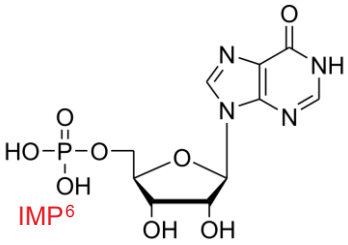


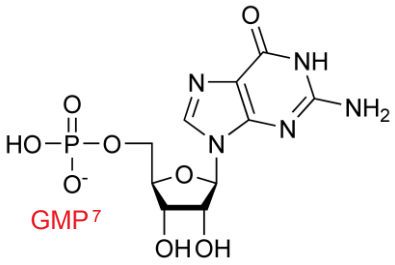




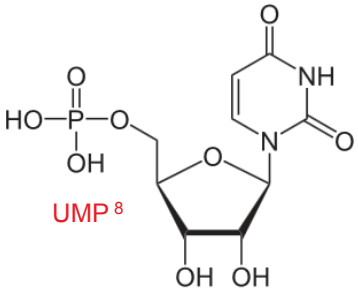


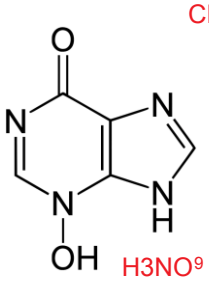


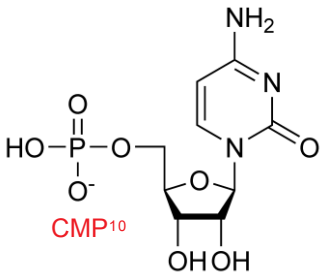




Avoidance-attraction neutral compounds:







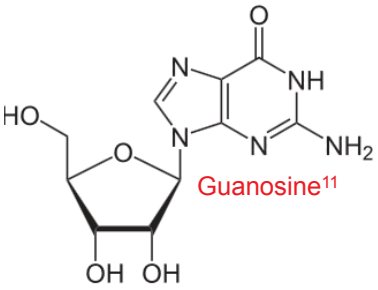


Figure C. Chemical structures of avoidance-attraction inducing and avoidance-attraction neutral compounds.

[1] By Pepemonbu (Made with BkChem and Inkscape.) [GFDL (http://www.gnu.org/copyleft/fdl.html) or CC-BY-SA-3.0 (http://creativecommons.org/licenses/by-sa/3.0/)], via Wikimedia Commons

[2] "Adenosin" by NEUROtiker - Own work. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Adenosin.svg#/media/File:Adenosin.svg

[3] "AMP chemical structure" by cacycle - Own work, ChemDraw / Photoshop.. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:AMP_chemical_structure.png#/media/File:AMP_chemical_structure.png

[4] "Adenosintriphosphat protoniert" by NEUROtiker - Own work. Licenced under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Adenosintriphosphat_protoniert.svg#/media/File:Adenosintriphosphat_protoniert.svg

[5] "Hypoxanthin" by NEUROtiker - Own work. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Hypoxanthin.svg#/media/File:Hypoxanthin.svg

[6] "Inosinic acid structure". Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Inosinic_acid_structure.svg#/media/File:Inosinic_acid_structure.svg

[7] "GMP chemical structure". Licensed under CC BY-SA 3.0 via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:GMP_chemical_structure.png#/media/File:GMP_chemical_structure.png

[8] "Uridinmonophosphat protoniert" by NEUROtiker - Own work. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Uridinmonophosphat_protoniert.svg#/media/File:Uridinmonophosphat_protoniert.svg

[9] "Hypoxanthine 3-oxide" by Yikrazuul - Own work. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Hypoxanthine_3-oxide.svg#/media/File:Hypoxanthine_3-oxide.svg

[10] "CMP chemical structure". Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:CMP_chemical_structure.png#/media/File:CMP_chemical_structure.png

[11] "Guanosin" by NEUROtiker - Own work. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Guanosin.svg#/media/File:Guanosin.svg