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REVIEW

Two-current choice flumes for testing avoidance and preference in aquatic animals

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Summary

1. Aquatic chemical ecology is an important and growing field of research that involves understanding how organisms perceive and respond to chemical cues in their environment. Research assessing the preference or avoidance of a water source containing specific chemical cues has increased in popularity in recent years, and a variety of methods have been described in the scientific literature. Two-current choice flumes have seen the greatest increase in popularity, perhaps because of their potential to address the broadest range of research questions.

2. Here, we review the literature on two-current choice flumes and show that there is a clear absence of standardized methodologies that make comparisons across studies difficult. Some of the main issues include turbulent flows that cause mixing of cues, inappropriate size of choice arenas for the animals, short experiments with stressed animals, failure to report how experiment and researcher biases were eliminated, general underreporting of methodological details, underutilization of collected data and inappropriate data analyses.

3. In this review, we present best practice guidelines on how to build, test and use two-current choice flumes to measure the behavioural responses of aquatic animals to chemical cues, and provide blueprints for flume construction. The guidelines include steps that can be taken to avoid problems commonly encountered when using two-current choice flumes and analysing the resulting data.

4. This review provides a set of standards that should be followed to ensure data quality, transparency and replicability in future studies in this field.

Key-words: avoidance behaviour, chemical ecology, chemically mediated behaviour, choice tank, ecotoxicology, flume, fluvarium, shuttle box, toxicology, zebrafish

Introduction

The field of chemical ecology investigates the production and release of chemosensory cues, and how organisms detect and respond to such cues. Traditionally, chemical ecology has been dominated by work on terrestrial animals (e.g. insects), while the aquatic realm has seen less research activity (Brönmark & Hansson 2000; Hay 2009; Brooker & Dixson 2016). There is, however, a growing research effort in aquatic chemical ecology that aims to advance our understanding of the role of chemosensory signalling in social interactions with conspecifics (Gerlach & Lysiak 2006; Derby & Sorensen 2008), interspecific interactions (Ferrari, Wisenden & Chivers 2010; Brown, Ferrari & Chivers 2011), orientation and navigation (Weissburg 2000; Atema, Kingsford & Gerlach 2002; Bett & Hinch 2016), water chemistry discrimination/preferences (Herbert *et al.* 2010; Jutfelt & Hedgärde 2013) and avoidance of toxicants (Cherry & Cairns 1982; Tierney *et al.* 2010). Disruption of chemosensory function by pollutants is rather well documented in aquatic organisms with reports of deleterious effects on feeding behaviour, predator avoidance, chemical alarm cue avoidance, and reproductive and social behaviours (Scott & Sloman 2004; Krång & Rosenqvist 2006; Krång 2007; Tierney *et al.* 2010; Olsén 2011). Additionally, direct avoidance of polluted water has been investigated (Cherry & Cairns 1982). More recently, similar functions have been studied in an ocean acidification context (Munday *et al.* 2009; Dixson, Munday & Jones 2010; de la Haye *et al.* 2012; Jutfelt & Hedgärde 2013; Sundin & Jutfelt 2016).

The methods used to measure behavioural responses to chemical cues have been necessarily diverse in order to accommodate a broad range of organisms and research questions. However, some of the variation in methodology can be attributed to a lack of guidelines on best practices, leading to the use of improvised and unvalidated methods, some of which have

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been criticized (Baird *et al.* 2014). For research questions in aquatic chemical ecology that focus on preference/avoidance responses of motile animals to water masses containing different chemical properties, a popular approach is to use two-current choice flumes (Atema, Kingsford & Gerlach 2002; Herbert *et al.* 2010). In this review, we propose best practices on how to build, test and use two-current choice flumes to measure behavioural responses of aquatic animals to chemical cues. We include design blueprints, validation steps, protocols for running experiments and reducing researcher bias, and guidelines for analysing data and interpreting results. We also discuss common problems and how they can be avoided. Our aim was to provide an across-discipline improvement in the quality and replicability of data arising from experiments using two-current choice flumes.

Chemoreception and olfaction

The terminology used in the chemoreception literature is sometimes confusing and thus warrants clarification. As humans, we have a certain perception of what separates different senses, but this may not apply to other animal groups (Marui & Caprio 1992; Hara 2011). In aquatic systems, water carries chemical signals, and in aquatic vertebrates like fish, these can be detected by the olfactory (odour) receptor cells, gustatory (taste) receptor cells and/or the distributed chemosensory cells (Hara 2011). Hence, taste and smell are recognizably different senses that respond to different environmental cues, involve different sensory organs, different parts of the brain, and often facilitate different behaviours, yet they both detect dissolved substances (Kotrschal 2000). Moreover, in invertebrates, the chemical milieu is detected by receptors that may not correspond to the vertebrate olfactory and gustatory systems (Schmidt & Mellon 2011). Indeed, crustaceans have two distinct chemosensory pathways, the olfactory pathway and the distributed sensory pathway, but their function and the type of behaviour they mediate sometimes overlap (Schmidt & Mellon 2011). Despite these points, many recent studies have assumed they are measuring responses to olfactory cues without evidence of the sensory system that is actually being tested (Munday *et al.* 2009; Dixson, Munday & Jones 2010; Jutfelt & Hedgärde 2013; Sundin & Jutfelt 2016). Because all the chemosensory systems of aquatic animals have the ability to detect dissolved substances at a distance from the source object, without direct contact with the source, it is not possible to know *a priori* the sensory system responsible for a given behavioural response to a chemical cue (Marui & Caprio 1992; Hara 2011). Therefore, we use the term chemoreception throughout this review as a collective term for all chemosensory pathways and recommend the same for future papers describing experiments where the active sensory pathway is unknown.

Methods for assessing behavioural responses to chemical cues

A variety of methods for assessing behavioural responses to chemical cues have been described in the scientific literature (Cherry & Cairns 1982; Rand & Petrocelli 1985). Early preference/avoidance tests were typically carried out using elongated counter-current channels that mixed and drained in the centre, with different water sources coming from each end of the arena (Fig. 1a). Perhaps the first such system was described in 1913, developed to study the responses of fishes to different levels of dissolved oxygen and carbon dioxide (Shelford & Allee 1913). Further developments led to the Y-maze, consisting of physically separated upstream branches into which the animal can move (Fig. 1b) (Chidester 1921; Ryback 1969; Castilla & Crisp 1970), and early versions of two-current choice flumes, which provide parallel laminar flows in a rectangular compartment termed the 'choice arena' (Figs 1g and 2) (Cherry & Cairns 1982; Rand & Petrocelli 1985; Winberg 1992). During the 1980s, a two-current choice flume system with video monitoring was developed and used to assess avoidance behaviour of fishes and invertebrates when exposed to waste water from a chemical plant (Randelov, Poulsen & Pedersen 1986). Other methods for presenting chemical cues include shuttle box systems (Fig. 1c) with two connected circular arenas (Serrano, Grosell & Serafy 2010), two-choice plume flumes (Fig. 1d), where cues are released as plumes (Gardiner & Atema 2007), and regular aquaria with cues released at point sources (Caprio *et al.* 2014). These methods each have their advantages (e.g. offering more natural settings, natural concentration gradients and plumes, testing which cues trigger and facilitate tracking behaviour, which cues are attractive from a distance), or they may simply be easier to build or operate. Each of these methods also has disadvantages, which may include unknown concentrations of the cues, poor control over cue dispersal, mixing of the water by the animals or physical barriers between cues. For many applications, the two-current choice flume provides the best behavioural apparatus for generating clear and unbiased measurements of preference or avoidance of distinct water masses with different chemical properties (Atema, Kingsford & Gerlach 2002; Herbert *et al.* 2010; Jutfelt & Hedgärde 2013; Sundin & Jutfelt 2016). Specifically, the main advantages of two-current flumes are (i) the cues are evenly distributed in each current, which remain distinctly separated throughout the choice arena, such that the concentration of cue in each half of the arena can be known, (ii) the separation of cues between the two sides is consistent, extends through the choice arena, and restores itself rapidly even when the moving animal creates turbulence and mixing, and (iii) the animal has immediate access to, and is in the direct view of the entire arena, which means that the animal is likely to encounter the two water currents repeatedly. In long counter-current tanks and Y-mazes, the movement of the animal is usually restricted in several directions, which can delay chemo-detection and complicate decision-making. Depending on the research question, a Y-maze set-up can be appropriate, such as when assessing a binary choice in upstream orientation (Krång & Baden 2004; Bett & Hinch 2016). For assessment of chemosensory search behaviour, such as attraction to and orientation towards pheromones or distant food items, flumes with longer arenas with distinct odour plumes can be used (Moore, Scholz & Atema 1991; Krång & Rosenqvist 2006; Dixson *et al.* 2014).

Two-current choice flumes are sometimes also referred to as two-channel avoidance flumes, fluvariums, Atema flumes or Loligo flumes. The method is, when properly conducted, reliable for the quantification of preference/avoidance of water with a given cue (Winberg 1992; Atema, Kingsford & Gerlach 2002; Herbert *et al*. 2010; Sundin & Jutfelt 2016). A two-current choice flume consists of an elongated rectangular tank with two laminar and parallel currents that remain separated throughout the choice arena despite the lack of a physical barrier, leaving the animal free to move between the two flows (i.e. the two sides of the choice arena; Fig. 2). The current is made laminar using baffles, grids and honeycomb collimator plates installed upstream of the choice arena. The water chemistry can be manipulated by adding cues to one or both of the currents, while simultaneously measuring behavioural responses to those cues. Experiments commonly involve assessing preference/avoidance for water low in dissolved oxygen (Herbert *et al*. 2010), high in dissolved carbon dioxide (Jutfelt & Hedgärde 2013), water containing toxicants (Cherry & Cairns 1982; Rand & Petrocelli 1985; Randelov, Poulsen & Pedersen 1986), cues emitted from predators (Leduc *et al*. 2004; Dixson, Munday & Jones 2010; Jutfelt & Hedgärde 2013; Sundin & Jutfelt 2016), from conspecifics (Gerlach & Lysiak 2006), from preferred habitats (Atema, Kingsford & Gerlach 2002; Gerlach *et al*. 2007; Gould, Harii & Dunlap 2015) or from food items (Dixson *et al*. 2014; Sundin & Jutfelt 2016).

Design and assembly of two-current choice flumes

Two-current choice flumes may be commercially purchased (e.g. Choice tank, Loligo Systems, Tjele, Denmark; Choice tank, Qubit Systems, Kingston, ON, Canada) or self-constructed. The blueprints provided in Figs S1–S7 (Supporting Information) can be used to guide construction of a two-current choice flume with a 20 × 20 cm choice arena. The blueprints are scalable and validated for the range 5 × 5 cm up to 40 × 40 cm arenas. Sizes outside this range may function as well, but like all flumes, require testing and validation before use in behavioural trials.

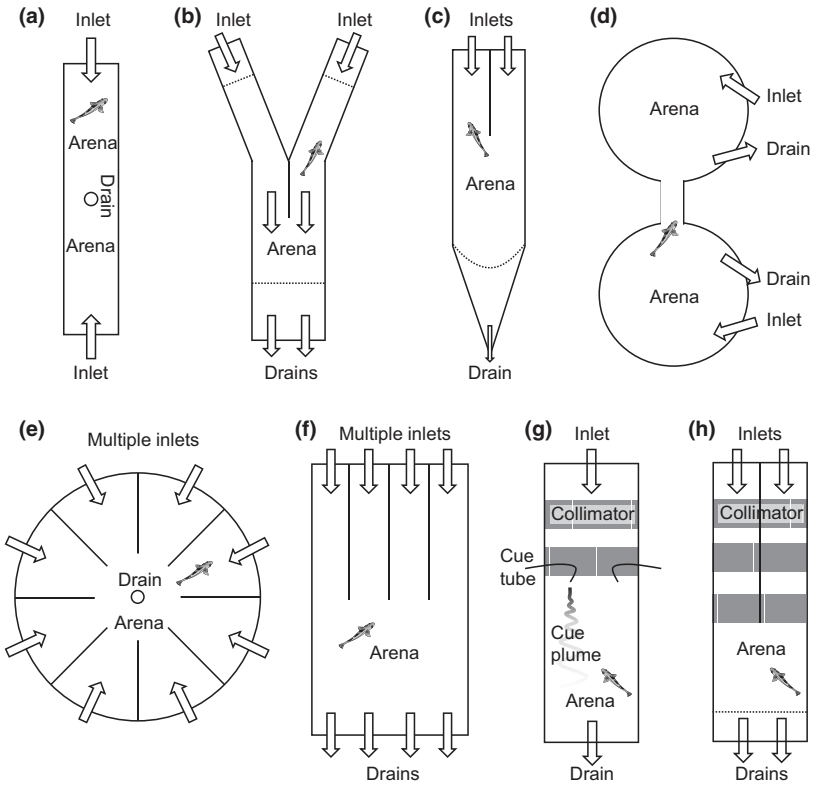

Fig. 1. Schematic overview of published methods for chemosensory avoidance and preference behaviour assessment in aquatic animals. Figures (a) through (d) are two-cue systems, while (e) and (f) can introduce several cues or concentrations simultaneously. (a) depicts a counter-current choice tank with inlets in both ends and a central drain (Shelford & Allee 1913). (b) is a Y-maze with inlets at the ends of the arms, a central arena and rear drains (Chidester 1921; Olsén 1985). The straight Y-maze (c) is similar in principle to (b), with the main differences being straight arms and a converging drain section (Gerlach & Lysiak 2006). Straight Y-mazes are generally small. The shuttle box system (d) consists of two circular tanks joined by a channel (Serrano et al. 2010). Slow circular currents keep the waters from mixing. Multi-channel choice tanks have been used for chemical gradients and multiple simultaneous cues, both radial currents with a central drain (e) similar to the counter-current choice tanks, and linear currents (f) similar to Y-maze flumes (Cherry & Cairns 1982; Rand & Petrocelli 1985). The plume flume (g) is similar to the two-current choice flumes, but has only one current. The cue is introduced into either side of the flume (e.g. via tubing) and spreads downstream, creating an odour plume that can be used for tracking (Gardiner & Atema 2007). The final apparatus (h) is the two-current choice flume that is the main focus of this review. All arenas are depicted from above and finer details (e.g. baffles) have been omitted for clarity.

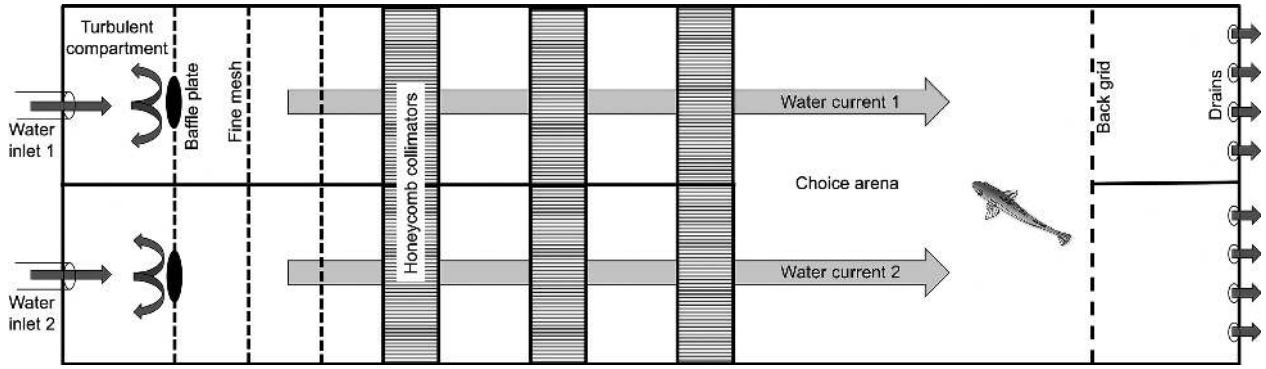

Fig. 2. Schematic of the general design of a two-current choice flume (overhead view). Water inlets are on the left, where baffles break up the concentrated flow and create turbulence, which helps distribute the flow evenly across the cross-sectional area of each channel. The downstream layers of mesh further even the distribution of flow, and honeycomb collimators create two separate currents of laminar flow that enter the arena where the animal is free to choose either current. By manipulation of the two water currents, behavioural responses to chemosensory cues can be determined. The water drains on the right end of the flume and is typically not recycled.

The size to which a two-current choice flume should be built depends on the size and motility of the study species. Large fish require choice arenas large enough to allow ample movement and reduce confinement stress (Atema, Kingsford & Gerlach 2002; Gardiner & Atema 2007) while still ensuring the arena is small enough that the animal can easily access each side (i.e. each current). For slower or smaller animals (e.g. many invertebrates), a smaller arena may be preferable as it will increase the ability of the animal to sample both water currents. Unfortunately, there exists no robust evidence on how to generate a rule of thumb for how large a two-current choice flume should be in relation to animal size and activity levels. More rigorous tests of optimal arena sizes for different animals are needed. Nevertheless, based on our experience with more than a dozen species of temperate and tropical fishes and crustaceans, we suggest that in motile animals (e.g. active fish), the width and length of the choice arena should each be around 4–15 times the length of the animal. To allow enough distance upstream of the choice arena for even, laminar flows to be generated, the total length of the flume should be at least three times that of the choice arena (Figs 2 and S1). The section at the rear of the flume should be designed so that there is minimal resistance to effluent water flow, which can otherwise create backflow of mixed water from the rear to the front of the choice arena. One practical issue to consider when choosing the size of the flume is the rapidly increasing water flow requirements with increasing flume size; it can be difficult to sustain flow-through in larger flumes.

Two-current choice flumes can be constructed using any inert material (e.g. acrylic or polycarbonate). Multiple layers of grids and honeycomb collimator plates upstream of the choice arena are needed to remove large-scale turbulence (Fig. 3), and experiments using flumes without any form of flow collimators may suffer from confounding turbulence and eddies (Supplementary video, part 7 – https://youtu.be/jrtyc-rLGWc?t = 360) such that animals may even be propelled 'upstream' (Baird *et al*. 2014). Plastic honeycomb collimator plates of different cell sizes can be purchased from companies such as Plascore (Waldlaubersheim, Germany) or Cel Components (Castenaso, Italy). Clearly, cell sizes of the collimators must be selected to ensure that experimental animals cannot pass through, as the addition of any mesh downstream of the honeycomb plate may introduce turbulence. At low water speeds (e.g. <1 cm s^{-1}) and particularly in small flumes (e.g. choice arena of $L \times W \times D = 4 \times 4 \times 2$ cm), honeycomb inserts are not always necessary. In such cases, several layers of fine mesh (e.g. ≤0·2 mm) at the inlets can be used to force incoming water to spread across the entire diameter of each channel (Supplementary video, parts 1 and 2 – https://www.youtube.com/watch?v = jrtyc-rLGWc). Each channel should then provide laminar flow to the choice arena as long as there are sufficiently long separated channels leading up to the arena (e.g. half the length of the entire flume).

As the upstream end of each channel is typically equipped with an inlet of a smaller diameter than the width of the channel, water is introduced to the flume through a concentrated jet. Even when honeycomb plates are being used, the jets of water entering each channel can create turbulence in the choice arena. Therefore, baffles must be installed close to the inlets, either using solid plates that force all of the incoming flow to move around the outside edges of the channel, or using a series of fine mesh screens (Figs 2 and 3a). These baffles serve to break up the jet and restrict any turbulence to the upstream-most end of each channel, while subsequent downstream collimators reduce turbulence and promote laminar flow (Herbert *et al*. 2010; Jutfelt & Hedgärde 2013; Sundin & Jutfelt 2016).

Flow rate and water speed

Two-current flumes can be sensitive to between-current differences in flow such that very minor differences in flow can prevent the two currents from remaining laminar through the choice arena. It is typically easiest to create laminar flows that remain separated through the choice arena at water speeds of around 1–2 cm s^{-1}, but the absolute speeds will depend on flume size, and should be validated for each flume and study species. Smaller flumes (e.g. choice arena of 4 × 4 cm) and

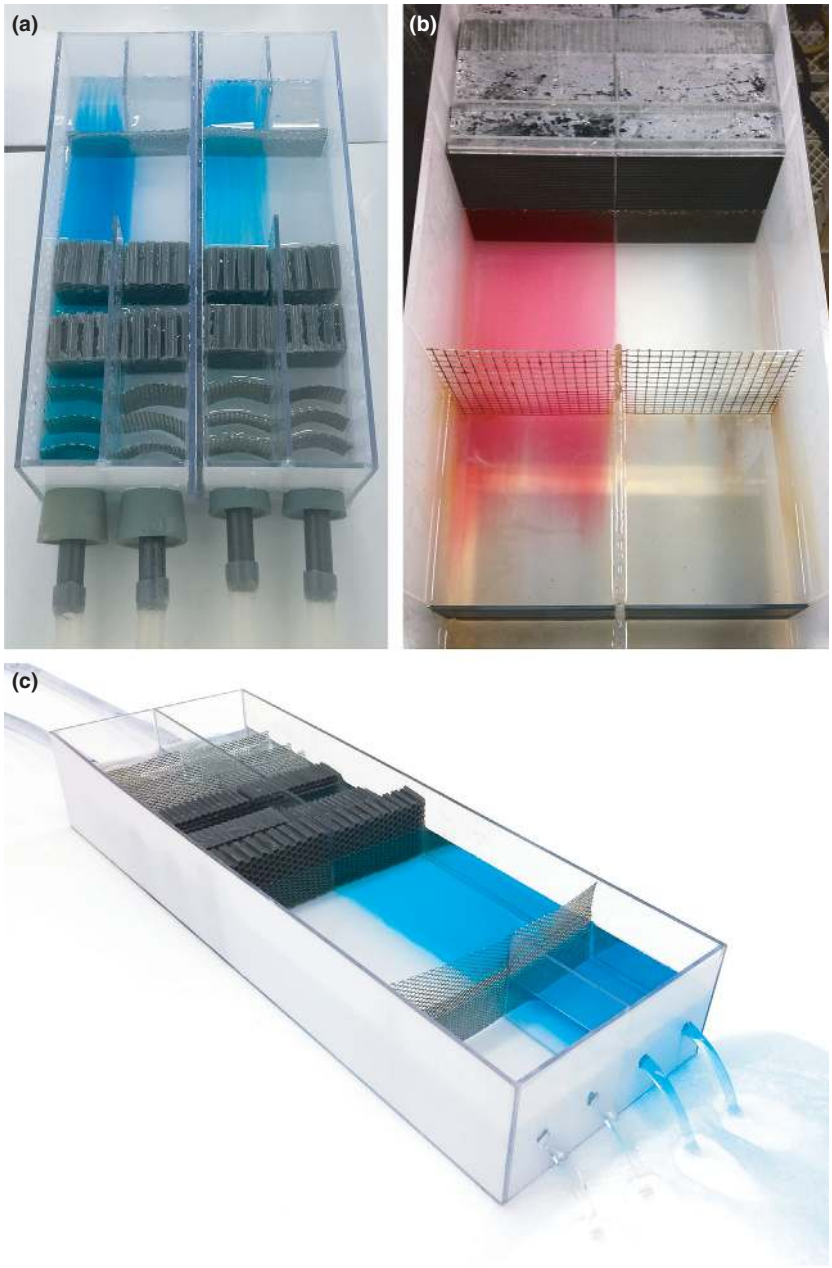

Fig. 3. (a) Photograph of two small (10 × 10 cm choice arena) two-current choice flumes pictured from above during a dye test using blue dye. The direction of water flow is from the bottom to the top of the picture. The flume has three layers of wire mesh with baffle plates on the first mesh, and two layers of honeycomb collimators to create laminar flow. The chemosensory choice arenas are the central squares, downstream from the collimators. (b) Photograph of a large (40 × 32 cm choice arena) two-current choice flume, where the direction of flow is from the top of the picture, and the red dye has passed the arena and is entering the drain area. (Modified from Sundin & Jutfelt 2016). (c) Side view of a dye test with a mid-sized two-current choice flume (20 × 20 cm choice arena). Videos of dye tests are available online: https://www.youtube.com/watch?v = jrtyc-rLGWc.

slow-moving animals may require slower water speeds (Baird *et al*. 2014), while large flumes and highly active animals may require faster speeds so that water separation is quickly restored after strong swimming bouts (see Supplementary video parts 13 and 14 – https://youtu.be/jrtyc-rLGWc?t = 835) (Atema, Kingsford & Gerlach 2002).

To generate stable flow rates in the inlet pipes, the water should be gravity-fed from a header tank with a constant head pressure (Atema, Kingsford & Gerlach 2002; Jutfelt & Hedgärde 2013). A simple way to ensure that water volume and head pressure stay constant in the header tanks is to allow some overflow in each header tank (Jutfelt & Hedgärde 2013). Flow meters and fine-scale flow controllers on inlet piping to the flume are usually necessary for generating equal flow speed on the two sides of the flume, particularly with decreasing flume sizes and flow rates. Flow meters can help to provide flow at a constant rate, but they do not regulate the flow to automatically compensate for any changes in water pressure, and need to be continually monitored so that minor adjustments can be made – a labour-intensive process. In this regard, flow controllers provide a more reliable way to ensure consistent laminar flow through the choice arena. Regular aquarium ball valves do not allow fine adjustments of flow and are therefore generally insufficient for fine flow control, particularly in small flumes.

In order to avoid mixing and overlap when the densities of the two water currents differ slightly (e.g. due to temperature or salinity differences), water speed can be increased. For example, a water speed of 6 cm s^{-1} was reported to allow two water currents with a 3 °C temperature difference to maintain

separation at the most upstream zone of the choice arena, while at the downstream end the colder water slid under the warmer water creating a central triangular area with stratified water that complicated preference attribution (Atema, Kingsford & Gerlach 2002). Obviously, care must be taken to ensure that water speed in the flume is well below the maximum swimming speed of the study species (see Baird *et al.* 2014). Differences in water densities can be substantial enough to create turbulence that cannot be avoided by adjustments of flume design or water speeds. For example, the density variation caused by a salinity difference of 6 PSU made laminar flow impossible in a flume with a 10 × 10 cm choice arena, regardless of water speed (Fig. 4). For choice experiments requiring differences in water density, shuttle box systems (Fig. 1) may provide more stable water separation (Serrano, Grosell & Serafy 2010).

Side switching valves

Test subjects can often show a preference for one side of the arena, independent of any difference in water chemistry. This can be due to small variations in the testing environment (e.g. uneven lighting, subtle differences in micro-turbulence) that may be imperceptible to the researcher, or be due to an innate side bias of the individual at the time of testing (Sundin & Jutfelt 2016). The best way to prevent side biases from influencing the experimental results is to switch the side of the cue-containing water during the testing of each individual. This can be accomplished by physically moving the tubing either between the two inlet valves on the flume or between the two header tanks (Atema, Kingsford & Gerlach 2002; Gould, Harii & Dunlap 2015). However, these techniques, particularly the former, are likely to startle the animal due to vibrations, and may interrupt the water flow or introduce air bubbles that may affect the behaviour of the animal. Instead, the switch of the cue water from one channel to the other should be done using switching valves on the inlet piping (Jutfelt & Hedgärde 2013; Sundin & Jutfelt 2016). The valves should be attached to a firm surface out of view of the test subject (Fig. 5) so that there is no physical or visual disturbance perceptible by the animal. The switch should be achieved by opening the second valve before the first valve is closed to ensure there is no interruption of water flow to the flume.

Supplementation of chemosensory cues

Manipulation of water chemistry may be achieved by adding cues and/or gases to one of the header tanks (mixing of water and cues/gases in the header tanks can be achieved using air stones) (Jutfelt & Hedgärde 2013). Experiments testing hypoxia avoidance generally bubble header tanks with nitrogen gas to reduce the partial pressure of oxygen (pO_2) (Herbert *et al.* 2010). However, large flumes with high water flow may require impractical amounts of nitrogen, and the solution can be to partially recycle the water to the header tanks for additional pO_2 control using air and nitrogen bubbling (Herbert *et al.* 2010). Having said that, complete flow-through is preferred to recirculating water when using two-current flumes, regardless of the research question. While the two water currents may be kept separated for extended periods of time when using recirculated water, there is always a risk of mixing. Indeed, mixing will be inevitable when switching which

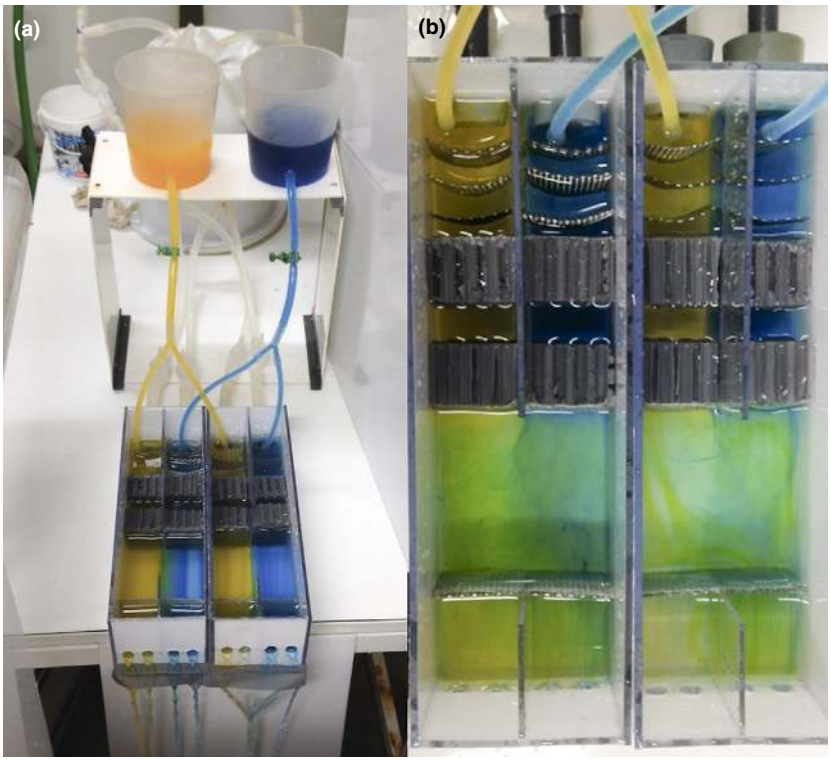

Fig. 4. Flume test using different salinities. Figure (a) shows a set-up with inlet tubing from two 100-L header tanks (not visible) and dye addition (yellow and blue small tubing and beakers) into the turbulent compartment of the flume, where the water from each of the two header tanks is of similar density. Figure (b) is the same set-up but with a salinity difference of 6 PSU between the two header tanks, showing that the laminar flow through the choice arena becomes severely disrupted and unusable for preference measurements.

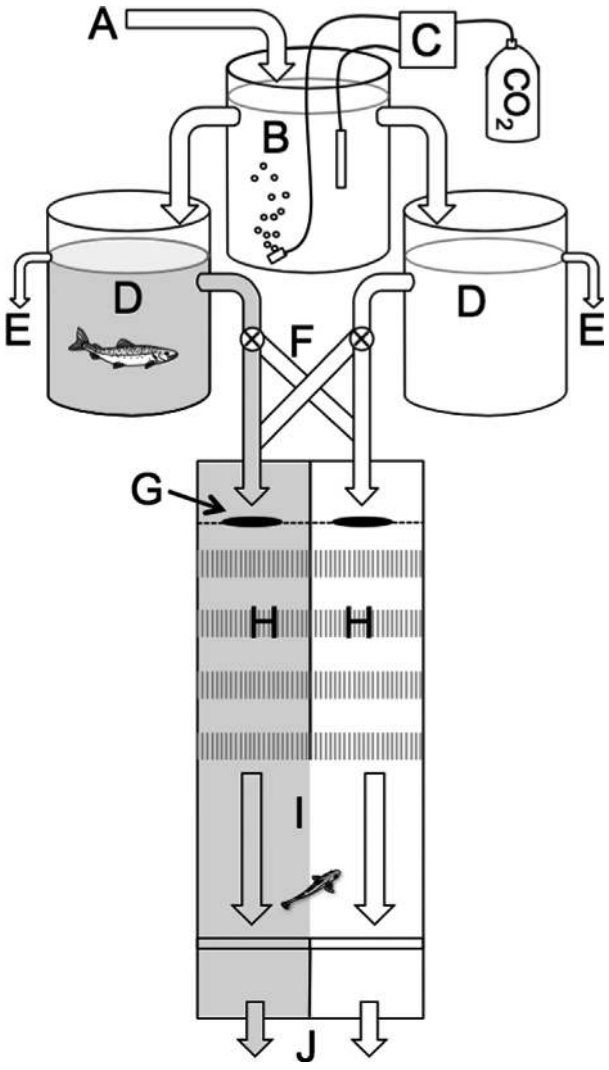

Fig. 5. Schematic of a two-current choice flume experimental set-up with control of CO_2 on both sides and fish chemosensory cues on one side (shaded areas). The letters represent the following: (A) Flow-through water inlet. (B) Main reservoir tank. (C) pH-stat system with pH probe and solenoid valve controlling the administration of CO_2 into the main reservoir tank. (D) Header tanks for the two sides of the choice flume, with one side containing a predatory fish. The reservoir and header tanks should be bubbled with air for mixing and oxygenation (not shown). (E) Overflow drains for the header tanks. (F) Cross-over piping for changing sides of the cue. (G) Baffle plate to break up incoming water jet. (H) Honeycomb plastic for laminar flow. (I) Choice arena for the test fish. (J) Flume drains (modified from Jutfelt & Hedgärde 2013). Not to scale.

channel the cue flows through, or because of turbulence caused by rapid movements of the test animal. Thus, recirculating the two outflows to the header tanks will lead to a reduced difference in gas or cue concentration between the two water sources as behavioural trials progress.

The easiest way to introduce cues from organisms is to keep the cue-releasing organisms (e.g. conspecifics, predators, prey, plants) in one of the header tanks (Fig. 5). This approach ensures that fresh cues are continuously released into the water flowing into the flume at levels that may approximate those that are ecologically relevant. For a semi-quantitative assessment of cue concentration, the number and weight of organisms in the header tanks, as well as water flow and header tank size, should be reported. However, the use of live organisms will generally preclude the potential to understand which cues are being released and/or detected. For example, a predatory fish will release a mix of chemical cues during its time in a header tank, and the mix may change over time. Immediately upon introduction into the header tank, the predator is often stressed from handling, which can cause the release of compounds such as mucus, ammonium, urea and cortisol (Dallas *et al.* 2010), as well as increased ventilation and oxygen consumption rates (Barton & Schreck 2011). Dermal abrasion from handling can also produce small skin scrapings that may cause release of cues detectable by the test animal in the arena; cues that will subside with time. An increase in oxygen consumption is accompanied by the release of CO_2, which could potentially be detected by the test animal (Caprio *et al.* 2014). Intermittent micturition and defecation by the cue-generating organism will also cause large temporal variations in cue composition and concentration. These issues may or may not affect the behaviour of the test animal; the solution is to aim for a steady state in which the cue-producing organism is acclimatized to the header tank. The effects of errors caused by temporal variation in cues should be minimized by alternating treatment groups from which the test subjects are drawn.

Eliminating experimenter bias

Objective measurements are absolutely vital to any scientific endeavour. All humans are susceptible to subconscious biases that can impede objectivity; biases that undoubtedly have affected the outcome of many published studies (Holman *et al.*

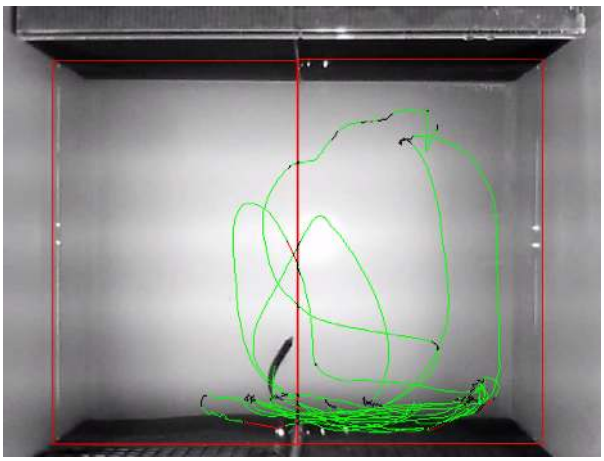

Fig. 6. The central choice arena seen through a tracking software (ZEBRALAB ViewPoint) with a goldsinny wrasse (*Ctenolabrus rupestris*) centrally in the bottom of the picture. The two red boxes were drawn using the tracking software and represent the two sides of the flume, one with and one without chemosensory cue. The track shows the movement covered during 1 min, and the different track colours depict different swim speeds (black = slow, green = intermediate, red = fast). The software quantifies time spent in each current, and how that changes over time (Modified from Sundin & Jutfelt 2016).

2015). When observing animals, our advanced ability for pattern recognition may work against us, and any preconceived ideas of the outcome of hypotheses, or the effects of treatments, may impede our ability to record animal behaviour objectively (Marsh & Hanlon 2007), and so do early tendencies in the results due to random clumping. For these reasons, it is essential to reduce the potential influence of observer bias. For direct observation of animal behaviour, the observer must be blinded to the treatment, as non-blinded observation may invalidate the results (Marsh & Hanlon 2007). Blinded observation can be achieved using codes, so that the person observing the animal (or a video of the animal) is not aware of the treatment or context of that animal. However, this approach often requires extra personnel, and the involvement of extra steps may increase the risk of making mistakes. In many experimental settings, particularly in limited-resource field-based trials, it is near impossible to keep the observer truly blinded to the treatment history and identification of the test animal.

Another approach, and the one that we strongly encourage, is the use of automated behaviour analysis systems (Sundin & Jutfelt 2016). These types of systems have been available since the 1980s (Rand & Petrocelli 1985). Here, behavioural trials are recorded with a camera placed above the flume (Fig. 6), and behavioural metrics are automatically quantified by software, either in real time or from recorded videos. There is a multitude of commercial (e.g. ZEBRALAB, ViewPoint, Lyon, France; LOLITRACK, Loligo Systems, Tjele, Denmark; ETHOVISION, Noldus, Wageningen, The Netherlands) and freeware (e.g. IMAGEJ, CTRAX, CASA, IDTRACKER, multiwell tracker) software that can be used to reliably track animal movement. Most useful in the context of two-current choice flumes is that many software packages enable automated quantification of the time spent by the animal in different zones (Fig. 6), facilitating assessment of how side preferences are influenced by chemical cues. A direct comparison between manual scoring of videos and automated video analysis showed that manual side scoring, such as noting the side each 10 s, can generate a similar result as automated video analysis (Sundin & Jutfelt 2016). Although manual scoring may provide similar results as video tracking, the automated software tracks with higher frequency and hence increased precision (Egan *et al.* 2009). An important advantage of video analysis is that the videos can be shared with other researchers. Additionally, notes that indicate the treatment details of the test animal can be displayed on video footage immediately prior to each trial commencing. Both of these steps greatly enhance transparency and reduce the potential for experimenter bias.

Methodological reporting and data analyses

Many published flume papers have provided insufficient detail to appropriately assess their methodology, which makes independent replication difficult. Some of the methodological details typically overlooked include (i) how blinded or unbiased measurements were achieved, (ii) how chemical cues were maintained and switched between sides during the experiment, (iii) what materials were used to baffle and collimate water flow in each channel of the flume, (iv) how 'unresponsive' animals were determined and how many were excluded from the data set, and (v) how the preference/avoidance data were analysed. While actual concentrations of chemical cues are not always straightforward to calculate, authors must provide the necessary details so that reasonable approximations can be made and compared across studies (J. Sundin, M. Amcoff, F. Mateos-González, G.D. Raby, F. Jutfelt and T.D. Clark, unpublished data). We urge researchers and editors to ensure videos of dye tests (with and without a test animal in the flume; see below) and flume trials are provided alongside the paper at the time of peer review and made publicly available online upon publication, so that laminar flow can be confirmed and the trials can be analysed independently by other researchers including via tracking software. It should go without saying that any images or videos of dye tests should represent the actual flume used in the study. We also encourage researchers to provide photographs and videos of their entire experimental set-up, including header tanks, side switching valves and flow meters/controllers. Gouraguine *et al.* (2017) elegantly demonstrate how video can be used to illustrate the flume methodology.

Several published flume papers also lack detail on data analyses, such as how the side preference was calculated and how it changed over the duration of the experiment, including a comparison of before and after switching the side of the cue. Whether collecting behavioural data from choice flume experiments using tracking software or manual recordings, side preference should be analysed and visualized over time (e.g. Sundin & Jutfelt 2016), rather than averaging the time spent in each water type across the entire trial. If this is impossible for some reason, cue preferences should, at a minimum, be presented and analysed as before and after the side switch, and illustrated using transparent data presentation standards (e.g. boxplots or histograms rather than bar plots; Weissgerber *et al.* (2015)). Assessing the effect of time within each trial provides information on (i) the time required for the animal to respond to the cue, (ii) response persistence, (iii) whether habituation to the cue occurs over time, and (iv) how the animal responds to switching the side of the cue (Fig. 7). In addition, presenting the data as a function of time, rather than binning all data from the entire trial, increases transparency and is important when assessing whether the test animal is in a dynamic state of post-handling recovery while the trial is being conducted. This approach is not restricted to the use of automated tracking software, as even repetitive manual observations can be analysed over time, for example by giving each observation on the cue side a value of 1 at each observation period (e.g. every 5 s) and then analysing the data in a binary format or calculating an average percentage of time on the cue side per 30 or 60 s. Scoring each individual as being either fully attracted or fully repelled to the cue based on where the fish spent >50% of its time over the entirety of the trial (e.g. Munday *et al.* 2010, 2013; Nilsson *et al.* 2012) is not recommended, as it removes important information, amplifies random effects, and could bias the results and their interpretation. For example, with

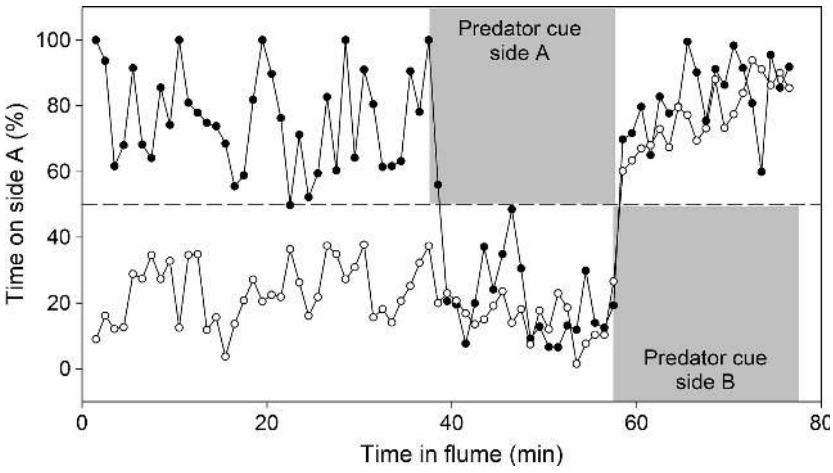

Fig. 7. Side preferences (1 min means) of two humbug damselfish (*Dascyllus aruanus*) over time. The first 38 min in the flume are without cue (note the different side preferences in the two individuals), then 20 min with a predator cue down one side of the flume (side A), and finally a side switch at 58 min before another 20 min with the predator cue down the opposite side (side B). The grey-shaded areas indicate the side of the flume containing predator cue.

this method two animals that spend 51% and 99% of their time in a cue, respectively, are scored as equally responding, whereas in reality the former likely lacks a preference response, while the latter demonstrates a very strong preference.

A mixed-model statistical approach is perhaps the most suitable for the data generated from choice flume experiments (Bolker *et al*. 2009). At a minimum, a model of choice flume data should include time spent in cue (seconds, proportion/percentage, or bimodal 1–0 preference–avoidance) as the response variable, trial time (e.g. minutes since start of the trial, removing a few minutes for the switch), treatment group (if applicable), the interaction between trial time and treatment as fixed effects, and the ID of each test subject as a random effect. Additional parameters of interest, such as animal size, sex and age (and potentially their interactions), can also be included in such a model, although if these parameters are not the primary factors of interest, entering them as random effects can simplify interpretation of the main treatment effects. Researchers should consider using cue side as an additional fixed effect, at least in initial models, to assess whether there exists a side preference unrelated to cue. If using the actual amount of time (e.g. seconds) the animal spends in a cue as the response variable, the use of a gamma distribution (see Zuur *et al*. 2009) may be necessary as there is a maximum time and therefore the time spent in the cue is not strictly continuous and unlikely to be normally distributed. Alternatively, if the response variable is formatted as a proportion or percentage (e.g. proportion of each minute spent in the cue), it will likely be appropriate in most cases to logit transform the response variable for use in modelling (see Warton & Hui 2011). It should be noted that using animal ID as a random effect may not control for all types of pseudoreplication within individuals. In some such cases, random slope models may be useful (Schielzeth & Forstmeier 2009), and although the application of random slopes may account for within-individual pseudoreplication with respect to the covariates to which they are applied (e.g. trial time), the main purpose of using random slopes is to allow the effects of covariates to vary by individual (or by group). When including time as a factor in a repeated-measures mixed model, the assumption of independence could be violated because of temporal autocorrelation within individuals [i.e. if an individual's side preference at time $t + 1$ is correlated with its preference at time t; Zuur, Ieno & Elphick (2010)], an issue that can increase the likelihood of type I errors. The presence of temporal autocorrelation can be assessed using plots of autocorrelation functions and, if necessary, corrected using a variety of approaches such as via the inclusion of a temporal dependence structure in the model (discussed in Zuur, Ieno & Elphick 2010).

Practical advice for starting flume experiments

Once a flume has been assembled, validation and pilot experiments can commence (Table 1). Use filtered water or a fine mesh to prevent detritus from entering the system and blocking flow. Bubbles must be removed from tubing and honeycomb collimator plates. A neutrally buoyant dye, such as fluorescein, should be introduced to one water inlet to observe the speed and behaviour of the currents. Dye tests should be performed repeatedly on both sides (Figs 3, 4 and S1) to ensure that the laminar flow remains consistent over time. Behavioural experiments can begin once reliable laminar flow (that quickly re-establishes itself after disturbance) is confirmed (see Supplementary video – https://www.youtube.com/watch?v = jrtyc-rLGWc).

In order to get reliable choice measurements from an animal, it is crucial to allow sufficient time for acclimatization and to ensure that the animal samples both currents (e.g. Fig. 7). The duration required for acclimatization and for the behavioural trial is dependent on how rapidly the animal recovers from handling stress and how quickly it explores the arena. Because this varies greatly between animals, pilot experiments are necessary to validate the time needed for recovery of normal exploratory behaviour. While there is no standard method for assessing recovery of normal behaviour (i.e. uninfluenced by the handling stressor), it has been repeatedly demonstrated that handling stress affects subsequent behaviour and physiology in animals (e.g. Barton 2002), sometimes for days or weeks (Pickering, Pottinger & Christie 1982). In this context, it is notable that most recent experiments using two-current choice

Table 1. Practical guidance for setting up and conducting two-current choice flume experiments

Experimental component	Recommendations
Flume dimensions	• Choice arena must comfortably accommodate test species across length, width and water depth • Flume length $> 3 \times$ the length of choice arena • Flow required to generate laminar flow through the choice arena must not exceed comfortable movement speed of test species
Baffles and collimators	• Flume should contain baffles at proximal upstream end of separated channels to break up and evenly distribute inflow • Collimators should be positioned after baffles to reduce turbulence and generate laminar flow through the choice arena • Ensure bubbles and particulate matter are cleared from collimators and baffles
Header tanks	• Flow-through header tanks with consistent water volume • Chemosensory cue should be at least roughly quantified and reported, and kept as consistent as possible across and within treatment groups
Inlet tubes and side switching	• Flow meters can be installed in the inlet tubing, upstream of each flume channel • If head pressure varies over time in header tanks, flow controllers should be incorporated into inlet tubes • Valves should be arranged to allow side switching of flows from header tanks without disturbing test animals or interrupting flow to the flumes
Testing laminar flow	• Dye tests should be conducted prior to experiments to confirm laminar flow through the choice arena and quantify speed and behaviour of water currents • Additional dye tests should be conducted (and captured on video) multiple times per day to confirm laminar flow
Reducing stress and minimizing side preferences unrelated to cue type	• Install a visual screen that extends all the way around the flume and is the same on all sides • Minimize shadows and ensure lighting is as even as possible • Conduct pilot experiments to quantify the post-handling time required for the animal to achieve consistent, routine activity levels • Never disturb the animal
Removing experimenter biases	• Video monitoring is preferable to provide evidence of results • Identification cards with all necessary information can be displayed on uncut video immediately prior to experiment commencing • Use automated tracking software to quantify behaviour and side preference of test animals • Steps taken to ensure blinded observations must be detailed in the methods

flumes to understand preference/avoidance behaviours in fishes have provided only 2–5 min of post-handling recovery time and trials have been completed within 9–15 min. Physiological reference data are sometimes available for the experimental species or for a close relative; these data could be used to guide initial study design (cf. Begg & Pankhurst 2004; Welch *et al.* 2014). In any event, sufficient exploration of the arena by the test animal is an obvious prerequisite before attribution of side preferences can occur. At regular intervals, preferably daily, flumes should be thoroughly rinsed using warm water (avoid detergents) to reduce the risk of lingering cues. Experiments using cues with higher hydrophobicity may require more thorough cleansing protocols to remove substances attached to flume walls and collimators. The rinsing procedures should also be conducted before and after storage.

Conclusions

Aquatic chemical ecology is an important and growing research field. Assessments of preference or avoidance of water with specific chemical properties or cues have increased in popularity in recent years, perhaps most notably in the context of ocean acidification, and these experiments have often employed choice flumes. The two-current choice flume can be a powerful tool for generating new knowledge relating to preference or avoidance responses. We hope that this review will provide helpful guidance and set standards for these types of behavioural measurements, and encourage methodological quality control in future experiments to ensure independent replicability of results.

Authors' contributions

All authors contributed significantly to the ideas presented in this paper. F.J. wrote the first manuscript draft. The figures were created by F.J. (1-3 and 5-6) J.S. (4) and T.D.C. (7). All authors helped to revise and improve the manuscript.

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Data accessibility

This article generally does not present data. However, Fig. 7 shows results from a pilot study on two individuals, and the data can be downloaded from Dryad doi: http://dx.doi.org/10.5061/dryad.3r8n6 (Jutfelt *et al*. 2016).

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. 'Blueprints (Figs S1–S7) for constructing a two-current choice flume for testing avoidance and preference in aquatic animals' contains the descriptions of how to construct a two-current choice flume.

Video S1. Supplementary video.