Kinematics and behaviour in fish escape responses: guidelines for conducting, analysing, and reporting experiments

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Key words: biomechanics, fast-start, locomotion, whole organism performance, startle response, swimming, tracking

Abstract

Work carried out since the late 70s has provided key insights into the comparative biomechanics, kinematics, behaviour, and neurobiology of fish escape responses. With environmental change expected to affect the physiology and biomechanics of aquatic ectotherms, there is a growing interest in understanding how environmental stressors impact the swimming performance and behaviour of fishes during escape responses, particularly in the context of predator-prey interactions. As the study of fish swimming continues to expand, there have been repeated calls to standardise experiments and reporting practices to facilitate integrative and comparative studies. Here, we provide a set of practical guidelines for conducting, analysing, and reporting experiments on escape responses in fish, including a reporting checklist to assist authors undertaking these experiments. These resources will facilitate executing and reporting escape response experiments in a rigorous and transparent fashion, helping to advance the study of fish swimming in an era of rapid environmental change.

Introduction

Escape responses are high-energy swimming bursts used by fishes to escape predation and aggression (Box 1). Comparative physiologists and biomechanicists interested in fish locomotion have a long history of studying escape responses, starting with pioneering work based on hand tracings of body movements (Weihs, 1973; Webb, 1975; 1976). Steady improvement in methodology brought about by digital video recording, affordable high-speed cameras, and data extraction software has since increased the general interest in the study of escape responses, allowing important insights in fields including biomechanics, functional morphology, predator-prey ecology, muscle physiology, and neurophysiology (Domenici, 2011). In parallel to these developments, there has been a growing interest in understanding how anthropogenic stressors affect whole organism performance in fishes, particularly in the context of predator-prey interactions and climate change (Domenici et al., 2019).

Methods in experimental biology are increasingly employed to address interdisciplinary questions aimed at evaluating and predicting how organisms respond to human-induced environmental change (Stillman, 2019; Hof, 2021). However, there is also a pressing need to ensure that trainees and researchers venturing into new fields have access to resources allowing

them to understand and rigorously apply these methodologies (Killen et al., 2021; Roche et al., 2022).

Here, we provide practical guidelines for carrying out escape response experiments in fishes across fields spanning behavioural ecology, ecophysiology, biomechanics, and ecomorphology. We outline six important steps: considering important species-specific characteristics, designing an appropriate experimental setup and protocol, recording escape responses, extracting data from videos, analysing data, and reporting methods and results (Fig. 1). We also provide a checklist to assist researchers report their methods and results transparently and facilitate study replication and evidence synthesis.

Box 1. A brief overview of escape responses in fish

Escape responses are a type of startle response (i.e., a locomotor reaction) induced by a sudden, threatening stimulus. Fishes exhibit different types of startle responses (Domenici and Hale, 2019), including withdrawal, which involves motion only by a portion of the fish' body (e.g., an eel retracting its head into a refuge); freezing, in which the fish abruptly ends any pre-startle movement and then remains immobile but alert for a period of time; and escaping, which is characterized by acceleration of the entire body in response to the threat. Escape responses are caused by the contraction of anaerobic (i.e., fast-glycolytic) muscle fibers, typically resulting in a rapid (tens of milliseconds) bend of the body, often into a C shape, followed by burst swimming. Since escape responses involve short but rapid bouts of acceleration, they are often referred to as *fast start* escape responses. Here, we use the simplest term, "escape response", since all escape responses are fast starts (but not all fast starts are escape responses as fast-starts also include predatory strikes; Domenici and Hale 2019). From a mechanical standpoint, escape responses are unsteady swimming behaviours that involve transient body-caudal fin (BCF) locomotion (Webb, 1984; Domenici and Blake, 1997).

Escape responses by fish were originally considered as a highly stereotypic behaviour triggered by the activation of large reticulospinal cells called the Mauthner cells (or M-cells), and other related neurons in the hindbrain (Korn and Faber, 2005). We now know that escape responses are not highly stereotypic and exhibit a wide range of kinematics (Domenici and Hale, 2019). Escape responses also occur that are not controlled by M-cells: they are characterized by longer response latencies and lower locomotor performance than M-cell mediated responses (Domenici and Hale, 2019; Hecker et al., 2020). Escape responses generally comprise three kinematic stages, which were first described by Weihs (1973): *stage 1* – the preparatory stroke; *stage 2* – the propulsive stroke; and *stage 3* – a variable stage involving continuous swimming, coasting, and/or deceleration. Most research has focused on the first two stages, which are crucial for avoiding predation (Walker et al., 2005). However, more recent work has shown that stage 2 is not always present in an escape response (reviewed in Domenici and Hale, 2019). Nevertheless, being able to identify these stages is important for measuring both behavioural and kinematic components of escape responses (Table 1). Kinematically, stage 1 begins at the onset of the escape response and ends when the rotation of the head changes the direction of the turn (double bend responses) or the body stops bending (single bend responses). Stage 2 begins at the end of stage 1 and ends when the rotation of the head stops or changes the direction of the turn.

Fast starts (i.e., escape responses and predatory strikes) are often classified based on the shape of the body (C-shape or S-shape) at the end of stage 1. C-starts were initially considered synonymous with escape responses, and S-starts with predator strikes. However, recent work has shown that fish sometimes escape using S-starts and attack prey using C-starts (for a review, see Domenici and Hale, 2019).

The sequence of events in a typical Mauthner-mediated escape response is as follows: a fish perceives the threatening stimulus, the sensory neurons excite the Mauthner cell ipsilateral to the stimulus, and the ipsilateral Mauthner cell inhibits the contralateral Mauthner cell while also exciting the contralateral axial musculature. *Stage 1* corresponds to the fish's body bending as a result of muscle contraction. In this stage, the fish's head and tail move but there is little motion of the fish's centre of mass. *Stage 2* is characterized by a return flip of the tail, leading to forward acceleration of the body, although some thrust can already be produced during stage 1 (Tytell and Lauder, 2008). The onset of stage 2 is not directly activated by the Mauthner cells (Domenici and Hale, 2019) and stage 2 is not always present in an escape response: it occurs in *double-bend* responses but not in *single-bend* responses (see Domenici and Hale, 2019). In *stage 3*, when it is present, the fish can continue to swim, coast, or decelerate, depending on factors such as the proximity of the threat and the surrounding environment. Given the inherent variability of this last stage, it is generally not considered in measurements of escape performance (see *Data extraction*).

Recent studies have shown substantial variability in the neural control, timing, and kinematics of escape responses among species, within species, and across contexts (Domenici, 2010b; Domenici and Hale, 2019). Escape responses can be S-starts or C-starts, and C-starts can include a single-bend (stage 1 only) or double-bend (stages 1 and 2). Turning rates during escapes can vary from slow to fast (Domenici and Hale, 2019). For a more detailed description of the diversity of escape responses in fish, including graphs of performance measures and tracings of body movements see Domenici (2010, 2011) and Domenici and Hale (2019).

Species-specific characteristics

Methods of measuring escape performance and the ease with which experiments are carried out are primarily influenced by differences in fish swimming behaviour and body size, both within and among species.

Swimming behaviour prior to an escape

Three broad categories of swimming behaviour can be considered for the purpose of escape response experiments: (i) continuous swimmers – fish that swim continually and often live in pelagic habitats; (ii) intermittent swimmers – fish that swim in a stop-and-go fashion and often live near the substrate or other structures in demersal habitats; and (iii) occasional swimmers – fish that spend most of their time immobile and often live in benthic habitats. The procedures to standardise a fish's position and motion prior to stimulation vary among these behaviour and habitat categories.

Continuous swimmers should be stimulated to elicit an escape response during constant, slow swimming, when fish are undisturbed. Some continuous swimming species do not behave naturally in aquaria, and a natural swimming behaviour can be induced by providing a gentle current (<0.5 L/s) in a circular tank or a flow tunnel against which fish can swim at a constant, slow speed (Marras and Domenici, 2013). Many continuous swimmers are schooling species and testing them in a school rather than individually might be more ecologically relevant (Webb, 1980; Domenici and Batty, 1997; Short et al., 2020).

Escape responses in intermittent swimmers should be triggered while they are swimming slowly or while still. The experimenter should standardize the swimming behaviour of these species prior to stimulation or account for variation in swimming speed in the statistical analyses (see Building s*tatistical models*).

Occasional swimmers tend to rest on the substrate and remain still for extended periods of time, often near or inside a shelter. Their position at the time of stimulation can be induced by creating a shadow in a small area of the tank or by using a refuge that can be lifted prior to stimulation (see *Stimulus type and operation*).

Body size

Fish body size must be considered when deciding on the size of the experimental arena and the frame rate of the camera used to record escape responses. Body size also affects key escape variables (Table 1 in Domenici and Hale, 2019). Unless size is a variable of interest in the study design (e.g., Domenici and Blake, 1993; Hale, 1996; Wakeling et al., 1999), researchers should aim to use fish of similar sizes across experimental treatments (e.g., within a body length range of 10%). A common practice to account for size differences among fish is to measure relative swimming speed in body lengths per second ($L \cdot s^{-1}$). Relative swimming speed can be useful for comparing results across studies; however, small fish are capable of much higher relative swimming speeds than large fish (Domenici and Blake, 1997). Therefore, absolute values should always be presented alongside measures of relative swimming speed.

Experimental setup and protocol

Arena size and shape

A key feature of any experimental setup to measure escape performance is the size of the experimental arena. The arena size must be sufficient for the test fish to engage in a natural swimming behaviour and escape at maximum performance when startled. If the arena is too small, proximity to the walls can affect the fish's reaction to the stimulus and impede its movements. The minimum distance between the fish and the arena wall at the time of stimulation should be at least two body lengths to avoid wall effects on swimming performance and trajectory (see Eaton and Emberley, 1991; Mirjany et al., 2011). In contrast, if the arena is too large, the time spent by the fish in the camera's field of view will be limited (see *Video recording*), considerably lengthening the time needed to conduct a trial. As a rule of thumb, the diameter of the arena should be between 6-10 times the body length of the test fish, with continuous swimmers requiring larger arenas than intermittent or occasional swimmers.

Arena shape is another important consideration when designing an effective experimental setup. Holding tanks and aquariums are often rectangular, offering corners where shy species or

individuals can hide and remain immobile. Circular tanks avoid this problem – although they do not eliminate problems associated with thigmotaxis (fish remaining close to or against the walls). If a circular tank is not available for species that tend to hide in corners, an acrylic sheet can be bent into a cylinder, fastened, and placed inside a rectangular arena (e.g., Gingins et al., 2017).

Water depth, temperature, and oxygen levels

Allowing a fish to display maximum escape performance requires that the experimental arena be free of physical and physiological constraints. Three key characteristics of the test water require consideration.

Water depth. The depth of the water in the arena should allow fish to swim without contact with the arena floor or the water surface. Restricting the water depth in the arena facilitates kinematic measurements in two-dimensions. A suggested rule of thumb is a water depth of 3-4 body depths for occasional swimmers and 4-5 body depths for intermittent or continuous swimmers. When the water depth exceeds this level, a mirror or an additional camera can be used to record vertical movements for kinematic analyses in three dimensions (see Supplementary Information) or to exclude trials with vertical movements above a certain threshold (e.g., one body depth; Roche, 2021).

Water temperature. Water temperature affects escape performance (reviewed in Domenici, 2010a) and should therefore be maintained constant throughout the experiments (within ± 1°C of the set temperature). A stable temperature should be achieved without disturbing the fish, by working in a temperature-controlled environment or via a continuous water exchange with an external, temperature-controlled water bath.

Dissolved oxygen (DO). Hypoxia can lower escape performance (Domenici et al., 2007), and DO levels should be maintained above 90% by bubbling air into the arena or water bath using an air stone. The air pump should be turned off or the air stone removed from the arena prior to stimulation to avoid disturbance.

Lighting and contrast

Extracting high-quality kinematic data from videos of escape responses requires that the experimental arena be moderately and homogeneously lit, creating contrast between the animal and the background. Light reflection on the water surface should be avoided as it creates glare that can interfere with the tracking of a fish's motion (see *Data extraction*).

Multiple flood lights (typically two to four) can be used to uniformly illuminate the experimental arena. LED lights are preferable to halogen lights that generate a lot of heat, and to neon lights that flicker. Flood lights should be placed above the arena, outside the arena walls, and facing down at an angle that avoids direct reflection into the camera lens. Uniform lighting can also be achieved by orienting the lights upward and illuminating a white panel above the arena. Alternatively, LED light strips can be placed around the top of the arena. Another option is to position light strips or floodlights below an experimental arena with a white bottom. This setup will illuminate the area around the test fish, creating a high contrast between the dark body of the fish and its white surroundings. The arena must be raised above the light source to achieve homogeneous illumination, and paper sheets can be positioned above LED (not halogen) lights to

act as diffusers. The arena background should provide an acceptable level of contrast between the fish's body and its surroundings to facilitate tracking.

To facilitate data extraction from recorded videos, the experimenter should test the tracking software on multiple sample videos before commencing data collection (see *Data extraction*). This step is important to modify the setup if tracking proves challenging or impossible (see Sridhar et al., 2019).

Acclimation to the experimental arena

Acclimation is needed when transferring a test fish from its holding tank to the experimental arena. The time required for proper acclimation can vary depending on the species and individual, and the handling procedure used for transferring fish. Work on cod (Artigas et al., 2005) and zebrafish (Ramsay et al., 2009) indicates that ventilation rates and cortisol levels return to control levels 60-90 minutes after fish are handled with nets, respectively. For some species, using a water-filled container rather than a dip net to avoid air exposure when transferring individuals might reduce stress levels and acclimation time (Brydges et al., 2009). Several escape response studies have used acclimation times between 30-60 minutes (e.g., Marras et al., 2011; Schakmann et al., 2021), but preliminary trials should ideally be carried out on a given test species to assess how different acclimation times affect individuals' responsiveness to stimulation. Similarly, the possibility of habituation or fatigue should be investigated when carrying out repeated stimulations to determine an appropriate rest period between trials (e.g., Jornod and Roche, 2015).

Stimulus type and operation

Various approaches can be used to elicit an escape response, including acoustic, mechanical, visual, and tactile stimuli. Information on each stimulus type and their pros and cons is provided in the *Supplementary Information*.

When operating a stimulus, regardless of its type, delimiting a restricted area of the experimental arena in which the test fish is stimulated facilitates video recording (see *Camera field of view*) and standardising the test fish's distance relative to the stimulus (Domenici and Batty, 1997). For mechano-acoustic or visual stimuli, placing the stimulus close to one of the arena walls helps position the 'stimulation area' towards the centre of the arena. If preliminary trials indicate that fish do not approach the pre-defined stimulation area 1-2 hours post release into the arena, slightly shading this area with the use of a mesh net can help induce proximity (Turesson et al., 2009). For occasional swimmers such as gobies, a shelter can also be provided in the stimulation area, which can be lifted prior to stimulation (Kimura et al., 2022).

Standardising the orientation of the test fish relative to the stimulus is important since body orientation can affect the perceived strength of the stimulus, escape directionality, and the fish's turn angle, which influences escape duration (Table 1; Domenici and Blake, 1993; 1997). Orientation relative to the stimulus ranges between 0-180°; it is calculated immediately prior to the onset of stage 1 (Box 1) as the angle between the straight line joining the tip of the snout to the fish's centre of mass (the fish's body axis) and the line joining the centre of the stimulus to the fish's centre of mass. Standardization can be achieved by stimulating fish only within a certain range of orientations relative to the stimulus (e.g., 60-120°) and/or including orientation as a covariate in the statistical model(s) (see *Building statistical models*).

Avoiding disturbances

Undesirable visual or auditory stimuli in the environment can affect how a test fish perceives and reacts to the stimulus – for example, by altering its responsiveness or response latency. To avoid disturbing the test fish, it is important to separate the arena and experimenter by an opaque partition or screen (e.g., Marras et al., 2011). If the camera used displays a live video feed, the experimenter can observe the fish's movements on an external monitor and operate the stimulus from behind the screen. When this is not possible, a small opening in the screen can allow the experimenter to observe the arena without disturbing the fish. Unwanted disturbance prior to stimulation, such as from the physical activation of the stimulus, should be avoided as it can bias response latency (see *Supplementary Information*).

Video recording

Camera position

When recording escape responses in two dimensions, the camera should be placed above or below the location of the stimulus delivery in the experimental arena (Fig. S1). The camera should be far enough from the area to minimize image distortion around the edges of the frame, or images should be corrected for distortion (e.g., with Matlab's undistortImage or fisheye correction available in most video processing software such as Adobe Premiere). The optical zoom should be used to record only the relevant section of the experimental arena (see below). Ideally, the camera can be controlled remotely. If not, it should be activated by the experimenter without disturbing the test fish. If a high-speed camera has a limited amount of recording time (i.e., due to loop recording) and/or cannot be controlled remotely, it is advisable to position the camera in front of the set up through a hole in the screen and record escape responses via a mirror angled at 45° above or below the experimental arena (e.g., Gingins et al., 2017).

The camera, experimental arena, and stimulus should not be physically connected to each other. This is to avoid the camera shaking when the stimulus is released, early stimulation of the test fish if the stimulus release mechanism is connected to the arena, or disturbing the test fish when replacing a battery or SD in a camera connected to the arena.

Camera field of view

The precision of the digitization process for extracting data from escape response videos is related to the precision of the digital image recorded by the camera in pixels. Therefore, high-speed cameras with a high resolution are preferred, and the optical zoom should be used to record only a specific area of interest within the experimental arena. The camera's field of view should be restricted to the 'stimulation area' (see *Stimulus operation*) since filming the entire arena reduces the resolution of kinematic measurements. The behaviour of the test fish beyond the 'stimulation area' can be monitored by an additional camera with a lower temporal resolution (e.g., 30 Hz). Ideally, the width and height of the camera's field of view should be approximately 4-5 times the body length of the test fish (Fig. S2). A scale (e.g., a ruler or grid) is needed in this area to allow kinematic measurements (see *Data extraction*).

Camera frame rate

Most studies of escape responses are based on high-speed video recording with frame rates between 240-1000 Hz (the frame rate of standard video is 24-30 Hz). High frame rates are needed to capture rapid body motions which last only a few milliseconds. Ideally, the camera frame rate should be set to capture a minimum of five frames during stage 1 of the escape response (i.e., <20° resolution for a 90 ° turn), allowing measurements of instantaneous rather than mean locomotor performance. Instantaneous performance is akin to a snapshot in time throughout the escape response, requiring multiple camera frames. In contrast, mean performance is an average value that can be based only on two frames, one at the start and one at the end of the event. The number of frames recorded during stage 1 of the escape response depends not only on the camera frame rate but also on the duration and total angle of the turn performed by the fish.

The duration of the turn during stage 1 depends primarily on the size of the fish and the temperature of the water, with smaller fish and higher temperatures leading to faster turns, which require higher frame rates (Wakeling, 2006). For example, the average turning rate of a 5, 10, and 25 cm fish is around 5500, 3000 and 1500°·s^{-1}, respectively (Domenici, 2001). Therefore, a typical 90° turn for fishes of these sizes will produce approximately 4, 7, and 14 frames if recorded at 240 Hz (Fig. S3). For this reason, frame rates of 500 Hz or higher are recommended for fish below 5 cm. Larval fishes typically require frame rates of 1000 Hz, whereas frame rates of 200-250Hz Hz are generally suitable for larger fish (>15 cm). Fig. S3 indicates the frame rate needed for a given angular resolution and for acceleration estimates during stage 1 as a function of fish size.

The temporal resolution of the camera will also affect the precision with which response latency can be determined. Minimum response latencies are in the order of 5-20 ms (Domenici and Hale, 2019) and independent of fish size (Turesson and Domenici, 2007). Therefore, a minimum frame rate of 240 Hz is recommended for measurements of escape latency regardless of fish size. Table S2 lists examples of affordable high-speed cameras.

Data extraction

Identifying a fish's centre of mass

The point typically used as a reference for the measurement of distance-time performance variables (Table 1) is the centre of mass of the fish, the location at which forces are assumed to act. Several methods have been used to estimate the location of a fish's centre of mass (Fig. 2). One commonly used approach in biomechanical studies is to divide the fish's body into many small segments and calculate the centre of mass as the average location of the fish, weighted by the density of the different body segments. We provide R and Matlab code (Tytell, 2023) for three methods that follow this approach, allowing to calculate and track the true centre of mass, the volume centre of mass, and the area centre of mass (see *Supplementary Information*).

The "stretched-straight" method is another approach for calculating a fish's the centre of mass. This method is the least accurate from a biomechanical perspective (Fig. 2), but the simplest and the most ecologically relevant since predators tend to target the visual centre of mass on a prey's body (Webb and Skadsen, 1980; Walker et al., 2005). The experimenter identifies the location of the centre of mass on euthanized, rigid specimens when the body is stretched straight (see Supplementary Material) and tracks that point during the escape response. The centre of mass

can be physically marked on live fish prior to an experiment (e.g., with a small piece of reflective tape temporarily glued onto the body; Domenici et al., 2008) or digitally identified in each video frame by using a cubic spline algorithm (Tytell, 2023). The stretched-straight centre of mass is generally located approximately 0.35-0.40 L from the tip of the fish's snout (Webb et al 1978).

Escape performance variables

Early work on escape responses tended to focus on variables related to locomotor performance, such as swimming speed, acceleration, and turning radius (Weihs, 1973; Webb, 1975; 1976). Subsequently, it became apparent that non-locomotor (i.e., behavioural) performance is also critically important in affecting escape success, including responsiveness to the threat, the timing of the response, and the direction of the escape (Walker et al., 2005; Fuiman et al., 2006; Domenici, 2010a). Importantly, however, the influence of different escape performance variables on survival is likely to be context dependent and species specific (Domenici and Hale, 2019). Thus, the choice of variables to measure depends on the species and question(s) being investigated. Table 1 provides an overview of commonly measured escape performance variables.

Distance-time variables (Table 1) are often used to measure locomotor performance during an escape response, and include distance travelled as well as speed and acceleration (i.e., the first two derivatives of distance with respect to time). Due to the process of taking numerical derivatives (see discussion in Van Breugel et al., 2020 and Walker, 1998), errors in distance measurements are compounded when calculating values of speed and acceleration: acceleration is the noisiest of the three variables, whereas distance travelled is the least noisy. For this reason, smoothing is recommended when computing maximum values of speed and acceleration (see *Smoothing*). Importantly, measurement errors are more likely to occur when extracting distance measurements from low-resolution (i.e., pixelated) images, which makes the centre of mass difficult to accurately identify and track.

Time frame of the analysis

Another important consideration when assessing distance-time variables is the time interval over which to take measurements. It is generally recommended to record distance-time variables within a fixed time rather than within kinematic stages because predator reactions are likely to be constrained by time rather than by the kinematic stages of the prey's escape response. For example, if one assesses maximum swimming speed based on performance values achieved by the end of stage 1, one would find that the greatest speed tends to occur during escapes with a longer stage 1, simply because of the longer time available to achieve high speeds in these responses. Similarly, large fish achieve higher speeds than small fish by the end of stage 1 because they take longer to complete this stage. Hence, as suggested by Webb (1976), a fixed time is typically used for measuring escape distance and maximum speed. This fixed time can be chosen as the average duration of stages 1 and 2 across all escape responses in a given group or study (Domenici et al., 2008). Maximum acceleration can be measured as the peak acceleration at any point in time during the escape response (Domenici et al., 2008).

Manual vs. automated data extraction

Measuring the motion of a fish's centre of mass can be done using manual or automated tracking methods. Manual tracking requires physically marking the centre of mass on test fish before running experiments – for example, by using elastomer tags or reflective tape (Domenici et al., 2008) – and is therefore restricted to tracking the 'stretched-straight' centre of mass. Manual tracking is more time consuming than automated tracking but has the benefit that it can sometimes accommodate low quality video (e.g., insufficient contrast, presence of glare disturbances) that complicates automated tracking solutions. Several options of free software are available to implement manual tracking, including the MTrackJ package in ImageJ (Meijering et al., 2012), Kinovea (www.kinovea.org), DLTdv (https://biomech.web.unc.edu/dltdv; available now without a Matlab license; Hedrick, 2008), and Tracker (https://physlets.org/tracker). The user must mark the centre of mass in subsequent camera frames, for example by clicking on its location, and the software outputs Cartesian coordinates (X and Y), which can be converted into kinematic measurements using an appropriate scale.

Automated tracking solutions are provided by computer vision techniques for object detection, which rely on algorithms to track objects through frame sequences (Dell et al., 2014). Numerous options exist in the computer vision literature, but often require a programming background to implement (Panadeiro et al., 2021). The DeepLabCut machine learning algorithm (Mathis and Mathis, 2020) and machine learning through DLTdv can automatically track many points on animal bodies without markers, and are becoming more user-friendly. Other freeware such as Kinovea (www.kinovea.org) and Tracktor (Sridhar et al., 2019) can automatically track single fish in noisy environments with minimal coding skills.

Smoothing

Smoothing, the process of removing noise or jitter from a signal to reveal an underlying trend, is essential to estimate velocity and acceleration during an escape response (Walker, 1998). Derivatives tend to amplify noise in measurements, and, without appropriate smoothing, can lead to incorrect results. Smoothing splines produce the smoothest possible curve through a set of points given a certain error and are recommended to estimate acceleration provided the approximate accuracy of a digitizing technique is known (Walker, 1998). It is also possible to use smoothing regressions (Lanczos, 1956) and low pass digital filters such as Butterworth or Chebyshev filters with cut-off frequencies corresponding to durations less than 10-20% of the stage 1 duration (e.g., if stage 1 is 50ms, then cut-off frequencies greater than 100-200 Hz are recommended). Finally, one can use running mean or running median filters with durations less than 10-20% of the duration of stage 1 (e.g., with a frame rate of 1000 Hz and a stage 1 of 50ms, the duration of the filter should be less than 5-10 frames long). Example code is provided in Tytell (2023).

Data analysis

Selecting response variables

Escape response experiments allow measuring numerous locomotor and non-locomotor performance variables (Table 1), and researchers are often interested in comparing these measures among species, populations, or treatment groups. Given that speed and acceleration are inherently noisy, they are generally considered less reliable measures of distance-time performance than escape distance (Domenici and Blake, 1997). However, both can have an

important fitness value because they relate to energetics, and therefore should be considered or analysed if a researcher is interested in muscle power or energy consumption (e.g., Walker et al., 2005). Performances measures that are recorded but not included in statistical models can be presented in a table for descriptive purposes (e.g., Roche, 2021).

Building statistical models

The choice of statistical model(s) to examine relationships (e.g., how does size or body/fin shape affect escape performance) or compare groups of fishes depends largely on a study's experimental design and the nature of the response variables examined. For example, whether each test fish is stimulated once (Domenici et al., 2008) or multiple times (Gingins et al., 2017) in an experiment affects whether repeated measurements must be accounted for. Repeated stimulations are often used in studies of escape response because obtaining multiple measurements increases the likelihood of observing maximum performance. Linear mixed-effects models can be used to accommodate repeated measurements on individuals and allow examining how multiple stimulus presentations affect performance – for example, through habituation (Marras et al., 2011; Roche et al., 2016; Roche, 2021). Most escape performance measures are expected to generate Gaussian (i.e., normal) error distributions. Exceptions are response latency, which can be bimodal (Domenici and Batty, 1997), and responsiveness and directionality, which follow a binomial distribution – these can be modelled using Generalized Linear Models. Variation in fish body size, motion (swimming speed), and position (distance and orientation relative to the stimulus) prior to stimulation can be controlled for by including these variables as covariates in the statistical model(s) (e.g., Gingins et al., 2017; Roche, 2021).

Methods and results reporting

Evaluating whether an experiment was rigorously conducted is only possible if the methods and results are transparently and comprehensively reported. Unfortunately, studies in experimental biology often suffer from a lack of reporting consistency and underreporting of methods and results (Clark et al., 2013; Marqués et al., 2020; Killen et al., 2021). To address this issue, we provide a checklist of key information that should be presented when authors report escape response experiments (Table 2). Reporting checklists are valuable tools to assist authors design and report studies; help editors and readers assess the reliability of a study's findings; and facilitate study replication and evidence synthesis such as meta-analysis (Parker et al., 2018; Killen et al., 2021).

Conclusion

Published studies of escape response experiments in fish are steadily increasing due in part to the greater accessibility of high-speed cameras but also to a heightened interest in how environmental stressors affect whole animal performance and predator prey-interactions (Domenici et al., 2019). As the study of fish escape responses continues to expand, standardising experiments and reporting practices is critical to facilitate integrative and comparative studies, as well as evidence synthesis. Rigorous methods and reporting practices strengthen not only the biological conclusions derived from a study's results, but also their transparency and reproducibility (Ihle et al., 2017; Aaron and Chew, 2021). The guidelines we provided here are intended to help researchers design, execute, and report escape response experiments,

particularly students and primary investigators entering this rapidly evolving field of research. Importantly, we acknowledge that our own previous work on escape responses is imperfect and contains design and reporting deficiencies. This perspective has been an opportunity to reflect on improvements going forward, and we hope that it will be as useful to others as it has been for us.

Data and code availability: The data and code to associated with this paper are publicly available at https://doi.org/10.5281/zenodo.7577129.

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Tables and Figures

Table 1. Performance variables measured during an escape response. Performance variables used to characterize an escape response can be broadly categorized into behavioural and kinematic variables. Behavioural variables describe non-locomotor components of the escape response, whereas kinematic variables describe locomotor components. Kinematic variables can be further categorized into distance-time variables and variables relating to manoeuvrability.

BEHAVIOURAL VARIABLES
Responsiveness. Responsiveness is a measure of whether a fish reacts to a threatening stimulus or not. Chronologically, this is the first variable that can be examined in an escape response. Responsiveness is recorded as yes/no for a given trial, and can be reported (but *not analysed*) as a percentage when stimulating individuals multiple times or when examining responses at the group level – for example when comparing the responsiveness of fish exposed to normoxia or hypoxia (Lefrançois et al., 2005). Low responsiveness has been shown to be a determining factor influencing the probability of prey being captured by a predator (Fuiman et al., 2006).
Escape latency. Escape latency (or response latency) can be measured as the time between the onset of the stimulus and the first visible response by the fish, typically the motion of the head initiating stage 1 of the escape response (see Box 1). When using a mechano-acoustic stimulus (e.g., an object falling inside a tube suspended over the water), the onset of stimulation is considered to occur when the object breaches the water surface. When using acoustic stimuli, the onset of stimulation can be measured based on signal synchronization with the camera use to record the escape response (Domenici and Batty, 1997). Measurements of escape latency are uncommon when using visual stimuli, except in the case of a light flash (Batty, 1989; Cade et al., 2020). Longer latencies are expected for visual than mechano-acoustic stimulation due to the longer neural pathways involved in processing visual information (Domenici and Hale, 2019).
Reaction distance. Reaction distance (also Flight Initiation Distance; FID) measures a fish's reactivity to a visual stimulus and corresponds to the distance at which the prey responds to an incoming stimulus. This variable can be measured in experiments using model or computerised looming stimuli that simulate an approaching threat (see *Stimulus type and operation*). Reaction distance tends to increase with the speed and the frontal size of the approaching object (Dill, 1974; Webb, 1986; Domenici, 2002; Cade et al., 2020). A related measure is the Apparent Looming Threshold (ALT), which relates the reaction of the fish to a threshold in the rate of change of the visual angle subtended by the predator's frontal profile onto the prey's eye (Dill, 1974; Webb, 1986; Paglianti and Domenici, 2006).
Directionality. Directionality indicates if the escape response occurs towards or away from the stimulus. Directionality is based on the direction of the head's motion relative to the stimulus. It and can be used as a proxy indicating whether the M-cell that fired was ipsilateral (in the case of away responses) or contralateral (in the case of towards responses) to the stimulus.

Away responses are those in which the stimulus is at an orientation ranging within 0-180° (from the fish's body axis) on the side opposite to the direction taken by the motion of the fish's head.

Escape trajectory. Escape trajectory is a circular variable ranging from 0-360° (Domenici et al., 2011) calculated as the angle between the line joining the centre of the stimulus and the fish's centre of mass at the onset of stage 1, and the body axis of the fish at the end of stage 2 (or stage 1 in single bend responses – see Box 1). It can also be calculated based on the fish's swimming trajectory using successive positions of the centre of mass towards the end of stage 2 (Domenici and Blake, 1993).

DISTANCE-TIME VARIABLES

Escape distance **(D_{esc})**. Escape distance (or cumulative escape distance) is measured as the distance covered by the fish's centre of mass (based on adding the distances between each successive x and y positions) over a fixed time, typically the average duration of stage 1 + stage 2 (Domenici et al., 2008).

Maximum swimming speed **(U_{max}).** Instantaneous speed (U) is the first derivative of cumulative distance and is simplest to approximate using a central difference algorithm (Hamming, 2012; Tytell, 2023). U_{max} is then computed as the maximum U achieved over a fixed time, typically the average duration of stage 1 + stage 2.

Maximum acceleration **(A_{max}).** Instantaneous acceleration (A) is measured as the change in speed of the centre of mass over time (i.e., the second derivative of cumulative distance). As with swimming speed, acceleration is simplest to approximate with a central difference method. A_{max} is also computed as the maximum A achieved by the fish over a fixed time, typically the average duration of stage 1 + stage 2.

MANEUVERABILITY VARIABLES

Turning angle. Turning angle is typically measured during stage 1 (i.e., stage 1 angle) as the angle between the straight line joining the fish's snout and centre of mass at the onset of stage 1 and the line joining the fish's snout and centre of mass at the end of stage 1.

Turning radius. Turning radius is measured as the radius of the approximate circle given by successive positions of the fish's centre of mass during stage 1. Domenici and Blake (1991) suggest using the simple formula $Turning\ Radius = d / [2 \cos (\pi-\gamma)/2]$, where d is the mean instantaneous distance travelled (i.e., distance covered between two successive camera frames) and γ is the mean instantaneous angle of turn of the centre of mass throughout stage 1. Turning radius tends to be a relatively constant proportion of body length, hence it is typically measured in Lengths (L).

Turning rate. Turning rate can be measured as the angular velocity of the straight line joining the fish's snout and centre of mass, and can be reported as an instantaneous (i.e., maximum) or

mean value (i.e., throughout stage 1). Turning rate is an important variable for avoiding predation (Walker et al., 2005), which is negatively affected by size (Domenici, 2001). In addition, turning rate can be a useful tool for distinguishing between "true" escape responses and "routine turns" by a fish. This can be done by running preliminary baseline trials in which the turning rate of spontaneously swimming fish is measured. True escape responses are characterized by a much higher turning rate than routine swimming turns (Domenici and Batty, 1997; Domenici et al., 2004; Meager et al., 2006). However, even for escape responses, turning rate can range from slow to fast (e.g., 900-1300 $°s^{-1}$ versus 1700-3000 $°s^{-1}$ in a 12.9 cm cod; Meager et al., 2006) and, in some species, turning rates follow a bimodal distribution (Domenici and Hale, 2019).

Table 2. A checklist of criteria for reporting the methods and results of escape response experiments in fish.

Information to report	Explanation and/or suggestions
Study animals	
Body length and mass of test fishes	Provide the mean, SD, and range for all treatment groups.
Swimming behaviour of test fishes	Fish can be classified as continuous, intermittent, or occasional swimmers in the context of fast-start experiments.
Experimental setup	
Dimensions of the experimental arena	Report the width and length for rectangular tanks or the diameter for round tanks or acrylic inserts in rectangular tanks.
Water depth in the experimental arena	Water depth must allow the full extension of the fish's fins without contact with the arena floor or water surface (ideally a minimum of 3-4 body depths).
Explain how the experimental arena was illuminated	Illumination needs to be homogeneous and can be achieved by means of LED flood lights or light strips placed above or below the experimental arena.
Position of the camera and its distance from the experimental arena	The camera can be placed above or to the side of the experimental arena, preferably at least one meter away.
Type(s) of stimulus used and operating mechanism	Stimuli can be visual, acoustic, mechano-acoustic, or tactile and rely on real or model predators.
How the onset of stimulation was identified and recorded (if applicable)	The onset of stimulation is typically recorded in the video of the escape response to measure escape latency to mechanoacoustic stimuli, or reaction distance (FID) in the case of visual stimulation.
How the arena was shielded from external disturbance	An opaque sheet or barrier should be used to shield test fish from visual disturbances.
Dimensions of the scale used for calibration for kinematic analysis	A grid or linear scale should be placed in the camera field of view, at the bottom of the experimental arena.
Experimental conditions	
How the water temperature was controlled	Ideally, the temperature range should be limited to T ± 1°C.
Mean water temperature and variation (e.g., SD or range)	In the holding tank(s) and the experimental arena(s).
Method used to avoid hypoxia in the experimental arena	Air saturation should be kept above 90%.
Frequency of water changes for closed systems	Water changes should be frequent to avoid a build-up of metabolites from test fishes (ideally, after each fish is tested).
Duration of animal fasting prior to stimulation	Fasting should be at least 24 hrs to standardize digestion prior to testing.
Experimental protocol	

Acclimation time to the laboratory (or time since capture for field studies) before starting the experiments	Acclimation time to holding tanks in the lab can depends on the species and method of capture/transport. Ideally it should be at least 48 hours.
Acclimation time to the experimental arena prior to stimulation	Ideally, acclimation should be at least 30 min, but will vary among species (likely to be longer for continuous than occasional swimmers).
Method used to identify and/or mark the centre of mass	Information on four common methods is provided in the Supplementary Information.
Camera frame rate and image resolution used	Both are influenced by the size of the test fish (see Fig. S3).
Number of repeated stimulations (if applicable)	Typically, 3 to 5 stimulations depending on the aim of the study.
Rest time between repeated stimulations (if applicable)	Rest will depend on the species and study aim, and should be investigated prior to the experiment. It should be similar to the acclimation time to the experimental arena.
Pre-stimulation variables such as the distance and angle of the test fish relative to the stimulus, and swimming velocity prior to stimulation	Report the mean and range (or SD) in the text or in a table. When variation in these variables is large (among individuals and/or treatment groups) they can be confounding factors if not controlled for in the statistical models.
Duration of the study	State the number of days needed to test all animals.
Data manipulation and analysis	
Any exclusions of test fish or trials from the analysis	Important for transparency and study reliability.
Criteria for excluding test fish or trials (if applicable)	Important for transparency and replication.
Statistical models and software used in the analysis	Reporting all models run, the variables included in the models, and the software used. Code-based statistical software promotes reproducibility by allowing code sharing.
Specify whether variation in body size was accounted for in analyses and describe any allometric body-mass correction/adjustment	Body size can be included as a predictor variable in statistical models (preferred option) or accounted for by dividing a performance variable by size (assuming isometric scaling).
Results	
Sample sizes for all treatment groups	Important for evaluating the robustness of reported effects.
Absolute measures of swimming performance in addition to any relative measures	Relative measures can be useful for comparing results across studies, but small fish are capable of much higher relative swimming speeds than large fish.
Provide a table with descriptive statistics of performance variables not included in the statistical models	Many escape performance variables can be measured (Table 1), but only a subset should be examined with inferential statistics based on the research question. Other measured variables can be presented as means with a measure of uncertainty (e.g., SD).
Particle Image Velocimetry	
Field of view illuminated by the laser light sheet	May be different from the camera field of view.
For a pulsed laser: time interval between pulses	Affects the maximum flow velocity that can be estimated reliably.

PIV processing parameters, including initial and final grid size (in pixel and mm)	Important for evaluating the reliability of velocity estimates
Flow velocity smoothing parameters, if applicable, particularly if missing vectors were interpolated	Flow field smoothing can affect the strength of vortices detected. In general, we recommend that researchers do not smooth the flow fields or use minimal smoothing, and do not interpolate missing vectors.

Figure 1. The steps involved in conducting, analysing, and reporting escape response experiments in fish, with important considerations at each step. Considerations marked with an asterisk are detailed or expanded upon in the Supplementary Information.

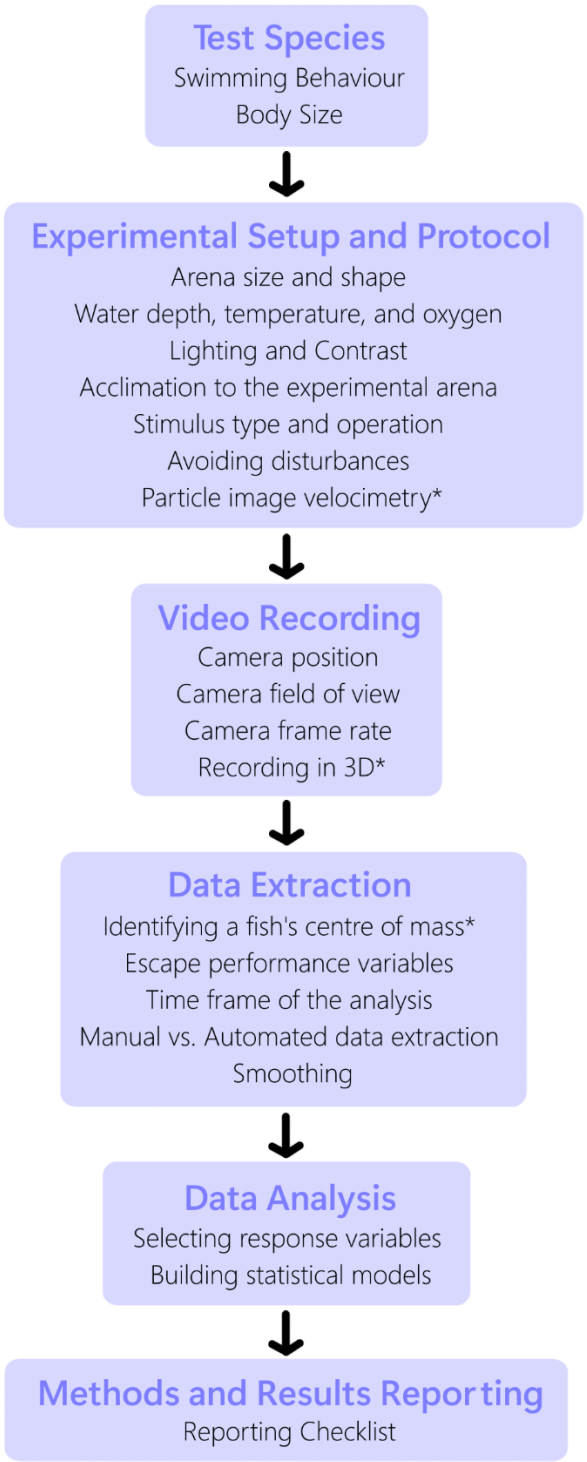

Fig. 2 A comparison of a fish's swimming speed (velocity) during the first two stages of an escape response using four methods to determine the location of the fish's centre of mass (CoM): the true CoM; the volume CoM; the area CoM; and the 'stretched-straight' CoM. Note that the true, area, and volume CoM can be located outside the fish's body when it is curved. The 'stretched-straight' method is the least accurate from a biomechanical perspective, but the simplest and most ecologically relevant as predators tend to target the visual CoM on a prey's body.