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

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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 ($L·s^{-1}$). 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 (π-γ)/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 $\pm$ 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.
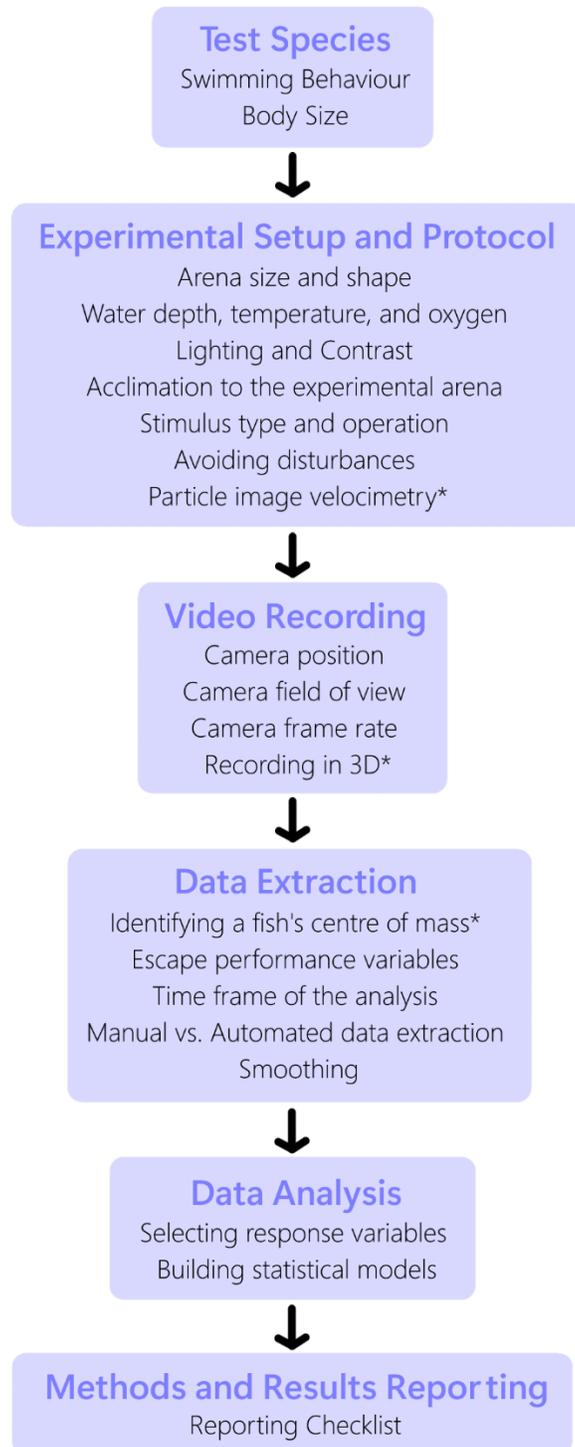
Fig. 2 A comparison of a fish's swimming speed (velocity) during the first two stages of an escape response using four methods to determine the location of the fish's centre of mass (CoM): the true CoM; the volume CoM; the area CoM; and the 'stretched-straight' CoM. Note that the true, area, and volume 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.
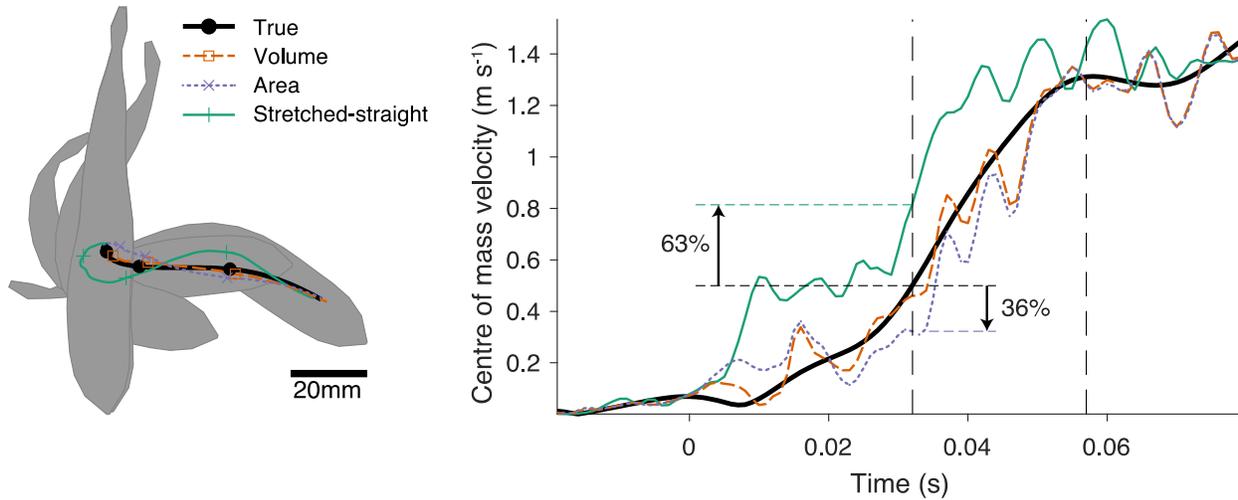
Kinematics and behaviour in fish escape responses: guidelines for conducting, analysing, and reporting experiments

Dominique G. Roche, Eric D. Tytell, Paolo Domenici

SUPPLEMENTARY TEXT

Types of stimuli to elicit an escape response

Acoustic and mechanical stimulation. Acoustic stimuli, either pure tone sounds (single frequency) or broad band sounds (multiple single frequency components), have been used to stimulate fish in numerous studies of escape responses (Domenici and Batty, 1994; Mirjany et al., 2011; Short et al., 2020). Using sound allows testing frequencies of sound reception, but does not always represent a natural stimulus, especially in the case of pure tones. A common alternative to using a purely auditory stimulus is the use of a mechano-acoustic stimulus, such as a tapered object (e.g., a weighted conical lab tube) falling into the water. This stimulus combines both a mechanical and an auditory signal that is picked up by a fish's lateral line and inner ear, respectively. The use of a falling object simulates the mechanical and auditory disturbance produced by an aerial predator and is therefore particularly relevant for fish that are preyed upon by birds. The tapered end of the lab tube reduces the disturbance at the water surface when the stimulus enters the water, which facilitates video recording. A falling object may also represent a visual stimulus; if the experimenter wishes to prevent visual stimulation while the object is falling, the stimulus can be made to fall inside an opaque PVC pipe prior to contact with the water surface (Gingins et al., 2017; Schakmann et al., 2021). The onset of the stimulation is thus considered to occur when the object breaches the water surface. Such stimulation can be assumed to correspond to a mechanical stimulation since visual stimuli result in longer latencies than mechano-acoustic stimuli (Batty, 1989; Domenici and Hale, 2019). Importantly, however, noise produced by the stimulus falling inside the PVC pipe can severely bias measures of response latency. Avoiding the stimulus hitting the sides of the PVC pipe while falling requires that the stimulus be centred inside a PVC pipe with a sufficiently large diameter (e.g., 3-4 times the diameter of the stimulus). The mechanism used to activate the stimulus, whether an electromagnet or a mechanical release, should also be silent. If the escape response is recorded with a camera positioned above the experimental arena (see *Camera position*), the timing of the stimulus can be recorded in the video by affixing a small mirror at approximately 45° on the arena wall nearest to the stimulus (see Fig. 1A in Schakmann et al., 2021).

Visual stimulation. Different visual stimuli have also been used to startle fish, including moving objects (Batty, 1989; Meager et al., 2006), flashes of light (Batty, 1989), and images produced by computer simulations (Paglianti and Domenici, 2006; Hein et al., 2018; Cade et al., 2020). Visual stimuli (with the exception of flashes) are typically not used to measure response latency but the distance at which an approaching object triggers a reaction in the fish (or the "effective distance" of a looming stimulus from a computer stimulation) and can be used to assess a fish's readiness to escape. Pioneering work by Dill (1974) found that the reaction distance to a visual

stimulus (also called Flight Initiation Distance; Ydenberg and Dill, 1986) increases with the speed and cross-sectional area of an approaching object (i.e., a looming stimulus). Light flashes are the only type of visual stimulus which have been used to measure response latency, which is typically longer in response to visual than mechano-acoustic stimulation because of a longer neural pathway (Batty, 1989; Domenici and Hale, 2019). Importantly, however, not all species readily respond to a light flash and the ecological relevance of this stimulus is unclear. Therefore, moving models used as looming stimuli are good alternative visual stimuli to a light flash, provided they are silent. Recently, several papers have used computer simulations of looming stimuli (Paglianti and Domenici, 2006; Hein et al., 2018; Kimura et al., 2022). Computer simulations are highly flexible because they allow readily changing the apparent speed of a looming stimulus (i.e., by changing how fast the image grows on the screen), and hence using speed profiles that reproduce the typical attack speed of different predators (Cade et al 2020). Simulations of looming stimuli have also been used to examine escape responses to multiple attacks (Kimura et al., 2022). The R package *loomR* allows generating looming stimuli on a computer screen for use in behavioural and neuroethological experiments (Carey, 2022).

Tactile stimulation. For certain species of fish and for larval fishes, visual or mechano-acoustic stimulation may not be effective. Instead, tactile stimulation of the head or the tail can be used to elicit an escape response (Liu et al., 2012). For example, a glass capillary tube can be used to make contact with the fish's body without bending or displacing it (Liu et al., 2012).

Real and model predators. Escape responses can also be triggered by means of a real or model predator. Model predators can be used to provide visual and mechano-acoustic stimuli, allowing to carefully control the predator's approach speed and behaviour (Meager et al., 2006; Stewart et al., 2014). In contrast, experiments with real predators offer less control but are potentially more ecologically relevant. Real and model predators can be used as a visual stimulus if separated from the test fish by a transparent divider (Meager et al., 2006), or in staged predator-prey encounters (e.g., Katzir and Camhi, 1993; Walker et al., 2005; Fuiman et al., 2006). Whether to use real or model predators largely depends on the research question at hand. If a study aims to examine how an environmental variable affects escape performance, then a model predator might be suitable. In contrast, if the objective is to investigate how escape performance or environmental stressors affect the probability of survival as a result of the effect on both predators and prey, then real predators may be the appropriate choice.

Being able to identify the precise onset of a stimulus delivery is important for measuring response latency, the time an individual takes to respond to a threat. As described above, measuring response latency is possible when using some but not all types of stimuli, depending on whether the stimulus is abrupt or progressive. The key to identifying the onset of a stimulus is to record it in the video.

Identifying a fish's centre of mass

Four methods can be used to identify a fish's centre of mass.

In biomechanical studies, the centre of mass is typically identified by dividing the fish's body into small segments and calculating the centre of mass as the average location of the fish, weighted by the density of the different body segments following the equation:

$$x_{COM} = \frac{\sum x(s_i)m_i}{\sum m_i} \quad \text{and} \quad y_{COM} = \frac{\sum y(s_i)m_i}{\sum m_i}$$

(eqn 1)

Where the horizontal midline of the fish is defined by points $(x(s), y(s))$, and s is the distance along the body from the tip of the snout to the tail.

(1) *True centre of mass*. Estimating the true location of the centre of mass requires having a measurement of the mass of each body segment from head to tail. The weight m_i in eqn 1 is the mass of each segment along the body (see Tytell and Lauder, 2008).

(2) *Volume centre of mass.* Few measurements of the mass of segments of fish exist to calculate the true CoM. However, many fish are close to neutrally buoyant. In this case, the mass of each segment is proportional to its volume. The cross-section of many fishes can also be approximated as an oval shape. Given these assumptions, m_i in eqn 1 is proportional to the volume of each segment: $m_i \propto w_i h_i$, where w_i and h_i are the width and dorso-ventral height of each segment.

(3) *Area centre of mass.* If a fish's body depth does not vary much along the body axis, a good approximation is to assume that the fish has the same height in every segment. In this case, $m_i \propto w_i$ in eqn 1, where w_i is the width of the body at position i from a ventral view (see Dabiri et al., 2014).

In ecological studies, the centre of mass is often determined using the "stretched-straight" method.

(4) *Stretched-straight centre of mass.* The estimation of the centre of mass of a fish when stretched straight can be done based on a subsample of euthanized, rigid individuals (Domenici and Blake, 1991). For laterally compressed fish, this position can be determined by hanging a dead fish (ideally when rigid, or frozen) from one point along its body profile. The procedure should be repeated for at least two (or three) different points; the crossing point of the two (or three) straight vertical lines descending from the hanging points will correspond to the centre of mass. For more elongate fishes, this position can be determined on euthanised rigid (e.g., frozen) individuals (Domenici et al., 2004). A long pin should be placed transversely through the body at various longitudinal positions (midway through the body depth), until the position at which the fish is balanced in the horizontal plane is found. This point corresponds to the centre of mass. A small number of individuals can be used to estimate the stretched-straight centre of mass (e.g., 3-5) since this measure exhibits little variation within species.

Fig. 2 in the main text shows a comparison of all four methods. The choice of method to determine the centre of mass will depend on the questions to be addressed. Work focusing on the ecological relevance of avoiding predation should use the stretched straight centre of mass,

3

whereas work focusing on biomechanics should aim to determine the true centre of mass (methods 1, 2 or 3). Using the stretched-straight centre of mass (method 4) leads to a substantial overestimate of velocity, particularly during stage 1. Therefore, methods 1-3 should be prioritized when comparing speed or acceleration during an escape response. However, the stretched-straight estimate can still be informative, particularly since predators tend to aim towards the visual centre of mass of the body (Webb and Skadsen, 1980; Walker et al., 2005), or if one is estimating distance travelled. R and Matlab code for all four methods is available in Tytell (2023).

Particle Image Velocimetry

Particle Image Velocimetry (PIV) is an engineering technique for quantifying water flow patterns. It is primarily used to measure two-dimensional flow velocities in a plane, but similar techniques have also been developed to measure three-dimensional flow velocities in a plane or throughout a volume. See Scharnowski and Kähler (2020) for a recent review and Tytell (2011) for a biological application of PIV in the context of fish escape responses. Materials needed to conduct PIV tend to be costly but inexpensive options for low-resolution PIV are also available (Ryerson and Schwenk, 2012).

Briefly, water is seeded with tiny (10-100µm) reflective, neutrally buoyant particles. Different types of particles can be used, including silver-coated glass beads (Tytell, 2006), cultured green algal cells (Ryerson and Schwenk, 2012), or dry artemia cysts (van der Hoop et al., 2018). For 2D PIV, the particles are illuminated with a light sheet, usually generated by a laser (although ultra-bright LEDs are becoming powerful enough, particularly for small arenas). The particles are then filmed with a high-speed camera and their movement analysed using PIV software. A key aspect of the PIV software's algorithm is that particle motion is analysed on a grid. Tracking average particle motion in a grid square has the advantage that the algorithm does not need to identify individual particles.

Once the flow field has been measured, the researcher can use standard fluid dynamic theory to estimate the fluid momentum, force, and power - see textbooks such as Smits (2000) and Batchelor (1973). For escape responses, which often begin from rest, the analysis may be relatively straightforward, relying on conservation of momentum. Assuming the effects of viscosity are relatively small, the momentum of the water surrounding the fish should be equal and opposite to the momentum of the fish (e.g., Tytell and Lauder, 2008). Alternatively, it is also possible to estimate force directly via algorithms that can estimate the pressure field from the velocity flow field based on PIV data (Dabiri et al., 2014; Lucas et al., 2017; Thandiackal and Lauder, 2020). Since pressure is force divided by area, once the pressure field is known, one can integrate (or add up) the pressure at each location over the surface area of the fish's body. Below are key considerations and rules of thumb for effective PIV measurements in fish escape response experiments:

Particle seeding density and grid resolution. The grid squares should be large enough so each square contains approximately10 particles (for the coarsest grid) but small enough that the

velocity of particles in the entire square is relatively uniform. Escape responses produce strong shear flows, which can make satisfying these requirements challenging.

Camera frame rate and shutter speed. For time-resolved PIV, experimenters often use a continuous laser and a high-speed camera. In this case, the shutter speed of the camera must be short enough that particles do not blur, and the frame rate must be fast enough that particles do not travel too far between frames. A good rule of thumb is that particles should not move further than 3-5 particle diameters between frames. The frame rate should also not be too fast because PIV can have a "peak locking" effect, in which velocities are biased toward integer pixel displacements. If the frame rate is too high, low velocities may be registered as zero.

Field of view. Pressure-based techniques (Dabiri et al., 2014), in particular, assume that the pressure field at the edges of the image is zero, which requires a relatively large field of view.

Shadows. Using multiple lasers (or splitting one beam) helps illuminate the fish from several angles and provides even illumination to avoid the fish's body producing a shadow.

Influence of laser light on fish behaviour. If possible, we recommend using infrared illumination (>800nm). In our experience, fish respond more naturally in these conditions than under the bright light of a visible laser, even if many species are capable of seeing and responding to infrared light (Matsuo et al., 2021).

Recording escape responses in three dimensions

Most fish species tend to swim and escape in the horizontal plane. Therefore, the study of escape responses has traditionally been based on a top or bottom view of fish motion, disregarding any motion in the vertical plane. While this is acceptable for most species, certain species can perform escape responses in three dimensions (3D), such as hatchetfish (Eaton et al., 1977) and knifefish (Kasapi et al., 1993). Other species such as killifish, also exhibit a vertical component in their escape response when stimulated from above (Fleuren et al., 2018). Therefore, the decision to analyse escape responses in 3D largely depends on the species investigated and the nature, position, and direction and of the stimulus relative to the fish. If the stimulus is directed from above the test fish, rather than from its side, and induces a vertical component in the response, 3D analysis is recommended. A simple method of accommodating 3D recording with a single camera is by filming from the side of the experimental arena and suspending a mirror at a 45 angle above the water surface (or below the experimental arena if using a tank with a glass bottom) to record horizontal and vertical motions simultaneously (Kasapi et al., 1993). Alternatively, two or three synchronized cameras can be used. In Fleuren et al. (2018), fish motion was reconstructed in 3D using a branch of Fish Tracker (MATLAB 2013, The MathWorks, Natick, MA, USA) as described in Voesenek et al. (2016).

Extracting 3D coordinates requires calibrating multiple cameras or camera views, which is done by collecting images of points with known locations in 3D space. This can be done using specialized calibration objects, black and white checkerboards (e.g., using the Matlab Computer Vision Toolbox), ChArUCO boards (similar to a checkerboard, but with each square labelled by

a pattern like a QR code; e.g., Karashchuk et al., 2021), or a wand (which has two points separated by a known distance; e.g., Theriault et al., 2014). Once calibrated, 3D coordinates can be triangulated from the camera views with a variety of software packages, including the DLTdv package (Hedrick, 2008; https://biomech.web.unc.edu/dltdv). DLTdv provides manual and automatic tracking options in two and three dimensions, and is available as a standalone package or as a Matlab add-on.

Recording measurements in the wild

Beyond the methods we reviewed above, developments in field cameras that can record in high speed are opening the door to the possibility of assessing fish escape behaviours in the wild (Hein et al., 2018). Laboratory experiments remain critical in studies of fish escape response biomechanics, neurobiology, and muscle physiology, but recording escape responses in the wild can be highly informative to determine how fish react to real or model predators in nature. In addition, while video recording remains a fundamental tool for measuring escape responses, other techniques are emerging, such as accelerometry, that can facilitate monitoring fish behavior in natural settings (Noda et al., 2014). Namely, by pairing accelerometry with high-speed video, it is possible to ow the detection of specific patterns of acceleration – for example, a predatory strike versus an escape from a predator (Broell et al., 2013). These acceleration "signatures" can then be used in the field to monitor fish locomotion as well as encounter rates with predators and prey, which represents a promising development for studies focusing on the ecology and the energetics of predator-prey interactions.

Table S1. The expected range of values for commonly recorded escape performance variables (adapted from Domenici and Hale, 2019). Plus ($^+$) and minus ($^-$) signs indicate variables that are positively or a negatively affected by fish body length. Indicative values are for body lengths sizes spanning approximately 5–50 cm. The range of maximum speeds is based on body lengths between 9.6–38.7 cm (Webb, 1976).

Escape performance	Variable	Values	Units
Behaviour	Responsiveness	0-100	%
	Response latency	5-150	ms
Kinematics	Escape distance (D_{esc}) $^+$	2-20	cm
	Escape duration $^+$	30-200	ms
	Maximum speed (U_{max}) $^+$	1.5–2.8	$m \cdot s^{-1}$
	Relative max speed (U_{max}) $^-$	3–30	$L \cdot s^{-1}$
	Maximum acceleration (A_{max})	20-150	$m \cdot s^{-2}$
	Turning radius	0.05-0.4	L
	Turning rate $^-$	500–8000	$° \cdot s^{-1}$

Table S2. Affordable high-speed cameras with frame rates and image resolution settings suitable for recording escape response experiments in fish. These cameras are available at the time of writing but will be replaced by newer models in time. Cameras with a lens that creates distortion (e.g., wide angle lens) should be avoided as these are not suitable for kinematic measurements.

Camera	Maximum frame rate	Price
Sony RX100	1000 Hz at 1080p	USD 1,200
Basler Ace (various models)	750 Hz, up to 2MP	USD 500-2,000

Fig. S1 Examples of experimental setups to study escape responses in fish. **A)** Side view: a mirror at 45° above the water surface allows recording the onset of mechano-acoustic stimulation in Schakmann et al. (2021). **B)** Side view: a mesh enclosure is used to help contain the movements of juvenile coral reef fishes to the camera's field of view; escape responses are filmed with a high-speed camera on a tripod (not shown) through a mirror positioned at 45° below the arena (Roche, 2021). The stimulus stand is fixed to a wall rather than the aquarium stand to avoid vibrations during its release. **C)** Side view and top view: a mesh net is suspended above the "stimulation area" to create shading and entice gobies to position themselves in the camera's field of view; the walls of the arena are angled to reduce thigmotaxis (Turesson et al., 2009). Images were reproduced with permission.
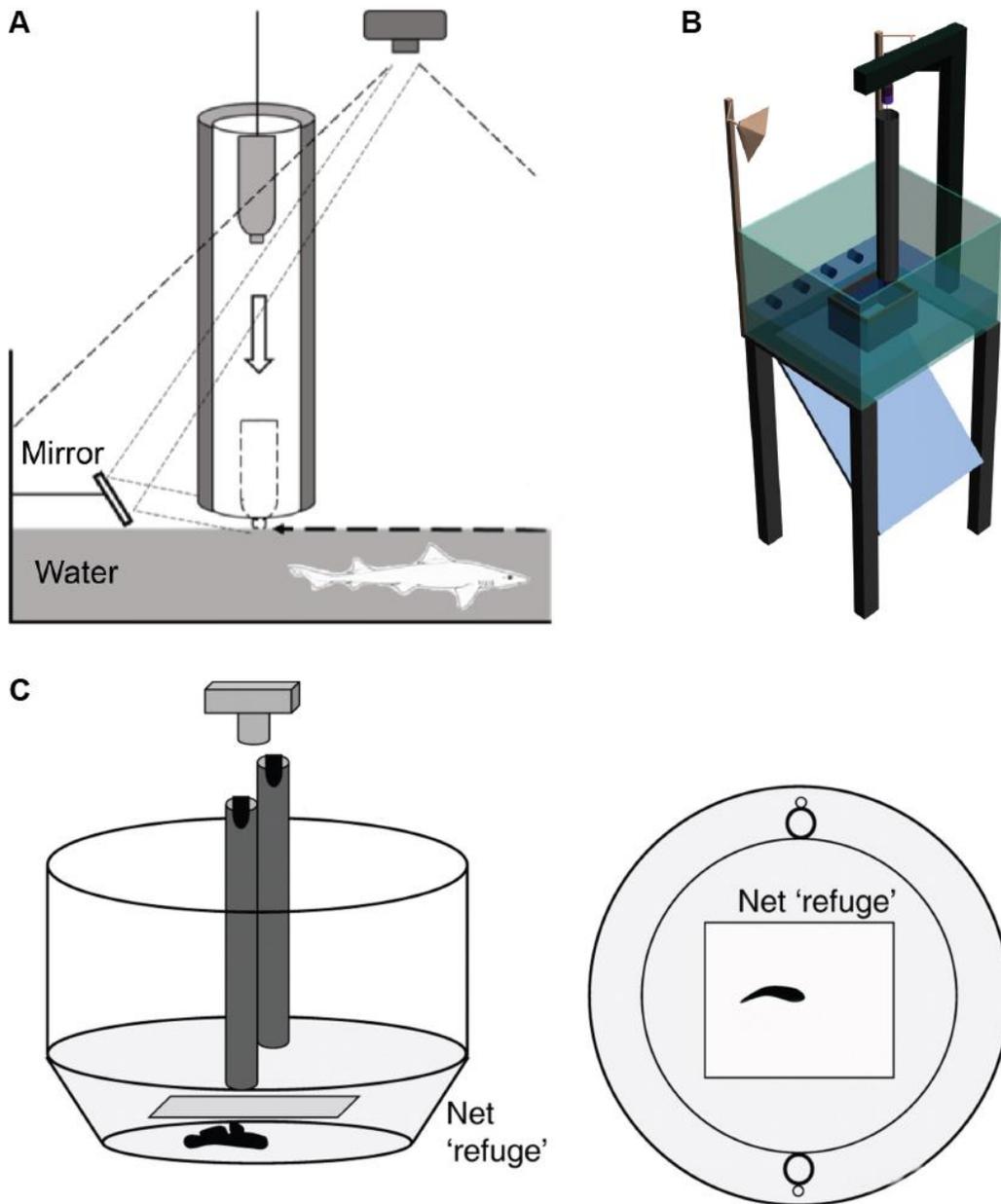
Fig. S2 An example of an adequate camera field of view (i.e., 4-5 times the body length of the test fish) to video record escape responses in the staghorn sculpin (*Leptocottus armatus*). The selected frames were recorded at 500 Hz and show the frame prior to the onset of stimulus (i.e. -2 ms before the stimulus hits the water surface), the onset of stimulation (0 ms), the end of stage 1 (60 ms), and the end of stage 2 (100 ms). The beginning of stage 1, at 30 ms, is not shown. The stimulus is a weighted lab tube (mechano-acoustic stimulus) released by an electromagnet, which falls inside a PVC pipe located in the bottom right corner of each frame. A small mirror positioned at 45° above the water surface, in the bottom left corner of the frames, allows identifying the exact moment when the stimulus breaches the water surface (at 0 ms).

- 2 ms

0 ms

40 ms

60 ms
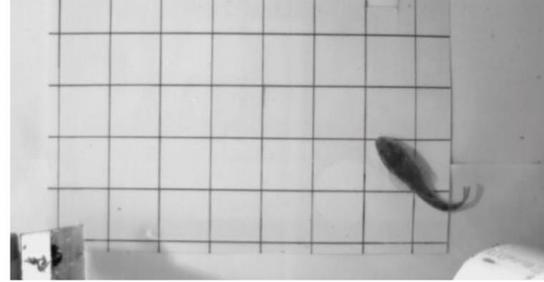
80 ms
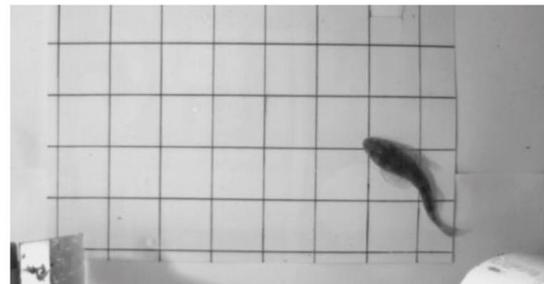
100 ms

Fig. S3 A-B) The camera frame rate needed to achieve an average minimum turning angle resolution of 20° (continuous line) and 10° (dotted line) per frame as a function of fish body length. Determined based on the relationship between average turning rate (i.e., turning angle divided by turning duration) and fish body length (Domenici 2001). Reference frame rates represent standard frame rates for high-speed cameras (60, 120, 240, 500, 1000 fps). **C)** Number of frames expected for a 90° turn as a function of fish body length at frame rates of 60, 120, 240, 500, 1000 Hz.

A

Fish total length (cm)	Frame rate for ≤20° res (Hz)	Frame rate for ≤10° res (Hz)
1-2	1000	2000
3-5	500	1000
6-14	240	500
15-33	120	240
37-79	60	120
>80	60	60
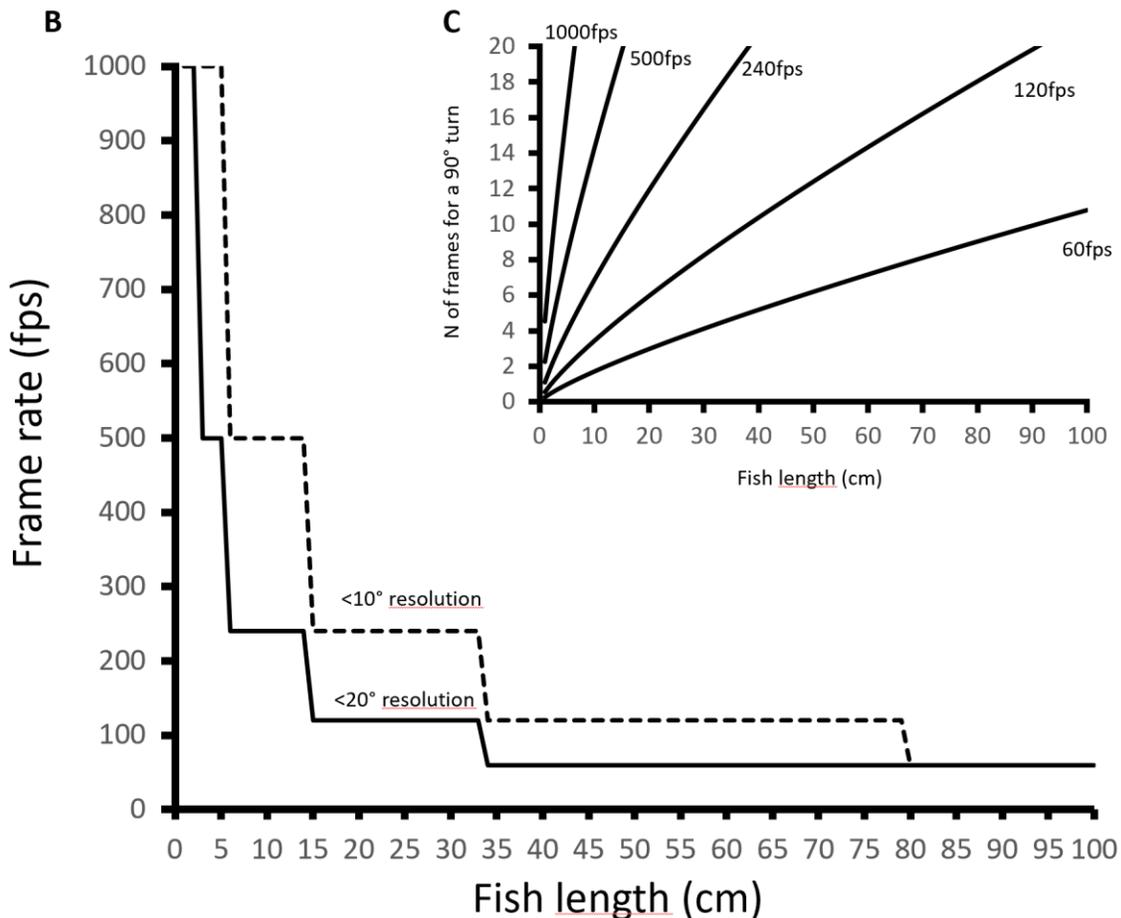
Video S1. An example video showing the escape response of a golden grey mullet *Liza aurata* (total length 14 cm) startled by a mechano-acoustic stimulus. The video was post-processed to increase the contrast between the fishes body and the background. The stimulus consisted of a tapered cylinder (10 cm long, 2 cm in diameter) dropped on the water surface, in the position indicated on the video by a black circle. The escape response was filmed using a high-speed camera (Redlake Motionscope) at 250 Hz. The video is played at 25 Hz (i.e., 10 time slower than the actual motion of the fish). The midline of the fish is shown in red during stage 1, and green during stage 2. The centre of mass (CoM) of the fish when stretched straight is shown as a circle along the body midline in each frame. The C-bend formed during the response is directed away from the stimulus. Escape latency is 20 ms. Maximum speed and maximum acceleration of the CoM are 1.4 m s^{-1} and 77 m s^{-2}, respectively. The average turning rate of the head (i.e., the segment from the tip of the head to the CoM) during stage 1 is 2,360 degrees s^{-1}. Reproduced from Domenici (2023), with permission.

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