A matador-like predator diversion strategy driven by conspicuous colouration in guppies

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Summary

Understanding the adaptive function of conspicuous colouration has been a major focus of evolutionary biology for much of the last century. Whilst considerable progress has been made in explaining how conspicuous colouration can be used in functions as diverse as sexual and social signalling, startling predators, and advertising toxicity [1], there remain a multitude of species that display conspicuous colouration which cannot be explained by existing theory. Here we detail a new 'matador-like' divertive antipredator strategy based on conspicuous colouration in Trinidadian guppies (*Poecilia reticulata*). Guppies encountering predatory fish rapidly enhance the conspicuousness of their eyes by blackening their irises. By pitting biomimetic robotic guppies against real predatory fish, we show this conspicuous eye colouration diverts attacks away from the guppies' centre-of-mass to their head. To determine the function of this seemingly counterintuitive behaviour, we developed a method for simulating escape probabilities when live prey interact with ballistic attacking predators, and find this diversion effect significantly benefits black-eyed guppies because they evade capture by rapidly pivoting away from the predator once it has committed to its attack. Remarkably, this antipredator strategy reverses the commonly-observed negative scaling relationship between prey size and evasive ability, with larger fish benefiting most from diverting predators. Taken together, our results introduce a new antipredator divertive strategy that may be widely used by conspicuously coloured prey that rely on agility to escape their predators.

Results and Discussion

Once detected and identified by their predators, prey must avoid being captured and killed. At this stage of the predation sequence many species exploit conspicuous colour patterns and other types of ornamentation to deceive or escape from their predators [2]. These can benefit prey by signalling their toxicity via aposematism [3,4], startling predators via deimatism [5], or by diverting attacks to expendable body parts [3,6,7]. The only well-established mechanism by which divertive antipredator strategies operate is based on ‘deflection’, whereby a predator’s initial strike, instead of incapacitating or capturing prey, instead makes contact with an expendable and conspicuous body part, allowing the prey to subsequently escape with its life [6,8–10]. However, whilst several studies have convincingly shown that predators can indeed be diverted towards conspicuous ornaments [11,12], except for some notable exceptions (e.g. [12]), surprisingly few have shown that this actually benefits live prey due to physical contact being made (as per the regular definition of deflection, reviewed in [6]). In addition, conspicuous colour patterns linked to survival during predator attacks are also found on body regions where damage could easily be fatal, for instance the white patch on the backs of rock doves which help them evade peregrine attacks [13], although the specific mechanism driving these benefits is unclear.

Using Trinidadian guppies (*Poecilia reticulata*) we demonstrate a new type of divertive antipredator strategy that, in contrast to deflection, requires a critically timed evasive manoeuvre from prey to be beneficial. Here, we show that prey use conspicuous colouration to direct attacks to a predictable location on their body, and once the predator has committed to its attack, the prey uses a ‘matador-like’ strategy whereby they execute a split-second escape response that takes them safely out of the predator’s diverted attack path. Remarkably, we show that guppies implement this strategy by enhancing the conspicuousness of their eyes via active colour change of their iris [14,15], which rather than directing attacks to non-vital body parts, actually directs predator attacks to their head.

Due to the inherent conspicuousness of the vertebrate eye [2], colour patterns that seem to mimic eyes are often incorporated into signals and traits designed to be maximally salient [7]. However, the function of conspicuous real eyes, found in multiple species across all vertebrate classes (e.g. [16–18]), is largely unknown. Indeed, many fish are capable of greatly increasing their eye salience via rapid colour change of chromatophores distributed across their irises [14,15,19], making them an ideal system for studying the adaptive function of eye colouration. In guppies and several fish species that blacken their irises (Figure 1A), this enhanced eye salience has been shown to honestly signal aggressive motivation to conspecifics [14,20,21]. Intriguingly, whilst predators are known to be highly attuned to eye-like objects [2,4], and black-eyed guppies are more conspicuous to predators at ecologically relevant distances (Figure 1B), we have also frequently observed wild guppies blackening their irises in the field when inspecting predatory fish in Trinidad streams (Video S1a & S1b). Inspired by these observations, we designed a series of experiments to reveal the function of this seemingly counterintuitive behaviour when guppies interact with predators.

Trinidadian guppies are a model system for testing the evolutionary context of anti-predator traits [22]. Waterfalls in the mountain ranges of Trinidad have restricted the upstream movement of predatory fish, creating replicated high- and low-predation risk guppy populations, leading to substantial divergence in a multitude of traits [22]. In lowland fish communities, ambush hunters are ubiquitous, and guppies are rarely out of visual contact of a predator at any one time [23,24]. As a result, guppies frequently approach and inspect detected predators to determine their predatory motivation [25] (Video S1b).

Using guppies caught from multiple streams across Trinidad that vary in predation risk, we first determined the ecological and evolutionary context in which different eye colouration is expressed when encountering predators. Second, to investigate how conspicuous eye colouration influences predator behaviour in isolation of any prey escape behaviours, we advanced on a recently-developed method for creating biomimetic model fish [14] and allowed natural predators to attack robotic guppies (whose colour patterns were calibrated to the predator’s visual system) that displayed different eye colours. Finally, to determine the adaptive function of this trait, we designed and validated an experimental paradigm that enabled us to calculate and compare simulated survival probabilities of live guppies based on their eye colour-associated escape strategies when they were attacked by real predators.

Eye colour of guppies under predation risk

We first assessed how eye colour expression varies in relation to predatory threat and evolutionary history by allowing individual guppies (n=250) originating from populations experiencing varying predation risk to inspect biomimetic models of pike cichlids (*Crenicichla frenata*). Pike cichlids are specialised ambush hunters that are particularly important predators of guppies [22], and guppies frequently approach and inspect this species to assess its predatory motivation [26]. To create our predator models, we printed photographs of a live pike cichlid onto thin sheets of polyvinyl chloride and adhered these to size-matched polyurethane casts of the same cichlid after it was euthanised (Figure 1C). The model cichlid colouration matched the variable visual systems exhibited by different guppy populations [27] due to being calibrated to the broad spectral peaks of our camera (see [14] for validation). In concordance with conspicuous black iris colouration having an antipredator function, we found guppies were considerably more likely to express black irises when inspecting the predator stimulus (both control and predator model) than when not inspecting (Binomial GLMM: χ^2=105.69, P<0.001; Figure 1E). When examining the sexes separately we found no significant predictors of eye colour variance in male guppies (Table S1). However, large female fish originating from rivers containing pike cichlids were more likely to express black irises when inspecting the predator models compared to the control stimuli (Binomial GLMM: predation history × predator stimulus, χ^2=6.62, P=0.010; predation history × body size, χ^2= 5.69, P=0.017, Figure 1E & 1F; Table S1).

These behavioural trials suggest large female guppies obtain the greatest benefit from displaying black irises when encountering pike cichlids. We hypothesised two ways in which this benefit may arise: (i) attack deterrence - black-eyed guppies deter predators, such as via pursuit deterrent signalling [28] or by increasing their perceived size, or (ii) attack diversion - conspicuous eyes divert predator attacks to the guppy's head, which enhances evasion when combined with the guppies' rapidly-pivoting escape response. Guppies, similar to most fish, escape predator strikes via the fast-start reflex, where attacked individuals rapidly pivot around their centre of mass before accelerating at a tangential angle to their original heading [29]. The success of this reflex depends on whether it is executed at the critical period whereupon a predator has committed sufficiently to its attack that it cannot react to its prey's sudden change of direction and acceleration [30]. Importantly, because a fish's centre-of-mass moves very little during the rotational stage of the fast-start and is the body part most susceptible to the propulsive forces generated by an attacking suction predator (i.e. the majority of predatory fish) [29], it is the usual target of predator strikes [31], whereas the head is the first body part to substantially move during this reflex. We thus hypothesised that guppies will be more likely to escape when fast-starting in response to a head-directed cichlid attack compared to an attack directed at the centre-of-mass. Because guppies take several seconds to blacken their eyes (Video S1c), it is unlikely the dynamism of the colour change itself is deimatic, i.e. functions to startle predators.

Predator responses to eye colour of biomimetic robotic guppies

To test our two hypotheses, we staged predatory interactions between live pike cichlids and biomimetic robotic guppies that differed in eye colouration. We first caught 52 wild pike cichlids from a mountain river in the Northern Range in Trinidad. Each cichlid was assigned to a pair of robotic guppies whose colour patterns were calibrated to a pike cichlid visual system ([14]; see Methods). Robotic guppies within each pair differed in eye colour but were otherwise identical, and were sequentially presented individually to each cichlid in a balanced order. Using an automated stepper-motor controlled system (Figure 2A), model guppies were programmed to simulate predator inspection behaviour and approach the pike cichlid whilst it was waiting for prey in its refuge. Cichlid attacks were filmed using an overhead high-speed digital camera (Video S1d).

Approximately half of the pike cichlids (27 out of 52) attacked one or both robots resulting in 35 out of the 104 presented robots being attacked (33.7%). We found no support for the deterrence hypothesis, since attacks were approximately equal between the two eye colours (17 vs 18 attacks on black vs silver irises; GLMM; χ^2= 0.28, P=0.60), and there was no difference between eye colours in the distance that the cichlids initiated their attack from (black-eyed robots: 36.3 ± 7.2cm vs silver-eyed robots: 33.7 ± 6.2cm (mean ± standard error); LMM; χ^2=0.06, P=0.81). Whilst previous work has suggested predator inspection behaviour may deter predator attacks [28], these results suggest eye colour does not provide additive information regarding the signalling component of this behaviour, perhaps because the characteristic swimming patterns of an inspecting guppy are sufficiently salient to communicate predator detection to a hunting pike cichlid.

To test the attack diversion hypothesis, we determined a cichlid's aiming point on a robotic guppy's body at two phases of the attack: the 'pre-acceleration phase' (just prior to the cichlid lunging), and the 'pre-contact phase' (just prior to physical contact). Pike cichlids visually track their prey before launching a ballistic attack ([32]; this paper), and cichlid aiming point was determined as the proportion position down a robotic guppy's body (0 = the tip of the nose and 1 = the end of the tail) that was bisected by a single line of symmetry projected from the pike cichlid's head (Figure 2B). We found that pike cichlid attacks on black-eyed robotic guppies were significantly anteriorly diverted compared to attacks on silver-eyed robots, which were attacked approximately at their centre-of-mass (pre-acceleration phase: aiming 18.3±8.0% (mean ± SE) along the length of the body at black-eyed versus 43.3±10.0% at silver-eyed robots; LMM: χ^2=5.27, P=0.022; pre-contact phase: aiming 19.5±3.9% along the length of the body at black-eyed robots versus 35.8±4.7% at silver-eyed robots; LMM: χ^2=9.11, P=0.003; Figure 2C).

Adaptive function of diversion

Our robotic trials show that conspicuous eyes significantly divert predator attacks, but nevertheless all the attacked robots were still engulfed by the cichlids (e.g. Video S1d), suggesting that rather than benefiting by deflection, the divertive effect must work in concert with the prey's escape response to be adaptive. To test this hypothesis, we developed an experimental paradigm allowing us to simulate the escape probabilities of real prey attacked by real predators. By isolating live guppies from live pike cichlids using a transparent barrier, we tracked the fast-start escape strategy of guppies (which guppies of all sizes perform) following a cichlid's ballistic attack and simulated and compared eye-colour associated survival probabilities. This method retains the biological realism that might be lost when using artificial stimuli to trigger fast-starts (where prey frequently fail to respond), whilst reducing ethical issues associated with staging live predation experiments between vertebrates [33].

We pitted trios of lab-bred female guppies (n=114) originating from a high-predation river population in Trinidad against individual lab-bred pike cichlids (n=12). During each trial, we continuously recorded the eye colour of guppies as they left a refuge to inspect the pike cichlid waiting in its shelter until they were attacked, again filmed using an overhead high-speed camera. Using the high-speed footage, we projected two lines denoting the eye-colour based simulated attack trajectories from the centre of the pike cichlids head until they bisected the guppy's body immediately prior to a guppy's fast-start. The bisection points were based on where cichlids aimed their attacks on our robotic guppies during the pre-acceleration phase of the attack (18% and 43% down their body length for black and silver-eyed fish respectively; Figure 3A). We used two bisection points to compare eye-colour based escape probabilities when the simulated and real eye colours matched, and the potential costs when they mismatched. We validated this approach by re-analysing footage from a previous study of live cichlid-guppy predation events [34] (Figure 4). The critical time period determining capture or escape once an attacked guppy initiates its fast-start in response to a predator's attack [34] will usually be shorter than the refractory period of the Mauthner neurons that control this reflex [35]. In most circumstances, guppies will therefore be incapable of executing a second fast-start before an attack outcome is determined, and our analysis of the live predation footage shows that, once their lunge has been initiated, cichlids do not alter their attack path in relation to the guppy's resultant escape trajectory, confirming that both attack and escape behaviours are ballistic. Using automated tracking, we calculated the shortest distances between the guppy's centre-of-mass (the body region a suction predator's gape needs to intercept to be successful [29]) and the attack trajectories throughout the fast-start sequence (Video S1e). Each frame was then scored as a 'capture' or 'escape' depending on whether the guppy's centre-of-mass was encompassed by the cichlid's gape. This capture/escape categorisation was then used as the binomial outcome in our statistical models.

In support of the attack diversion hypothesis, we found that diverting a predator's attack towards the head has substantial fitness benefits contingent on body size and the guppy's eye colour-associated escape strategy (Binomial GLMM; χ^2=6.37, P=0.012; Figure 3A-C). Guppies displaying black irises that anteriorly diverted a predator's attack increased their escape probability by an average of 38.8% compared to silver-eyed fish that were attacked at their centre-of-mass (Figure 3B, z=3.67, P=0.001). This increased escape probability was due to black-eyed fish escaping at more tangential angles during their fast-start compared to silver-eyed fish, allowing them to more quickly exit the attack trajectory of a lunging cichlid compared to non-diverted attacks on silver-eyed fish (t=2.94, P=0.024, Figure 3A & Figure 3E). This acute change in escape angle of black-eyed fish came at the cost of slightly reduced speed (LMM: χ^2=7.10, P=0.008, Figure 3D), consistent with previous demonstrations of a trade-off between these two kinematic metrics [29]. Our findings of eye colour-associated survival differences are supported by our reanalysis of live predation trials between pike cichlids and guppies from a previously published study [34]; here guppies had high survival if they escaped at a sharper angle in response to an anteriorly attacking pike cichlid, or escaped at a more parallel angle in response to a cichlid aiming more posteriorly (Figure 4).

Silver-eyed guppies showed an inverse relationship between body size and escape probability (Figure 3C), supporting theoretical expectations of locomotory scaling relationships where smaller prey have higher absolute acceleration and are thus more manoeuvrable and difficult to catch compared to larger prey [36–38]. This size-evasiveness relationship has important consequences for predator-prey interactions, such as predator hunting strategies [37,38] and size-dependent targeting by predators [32]. Remarkably, our results show that the diversion that results from predators attacking black-eyed guppies reverses this size-evasion relationship (Figure 3C). By increasing the distance between a predator's aiming point and the vulnerable centre-of-mass, our results show that escape probability increases with body size when black-eyed fish pivot away from a predator's diverted strike. In fact, the smaller fish in our study obtained no evasion benefit when displaying black irises, which when considered with the potentially increased detection costs of displaying conspicuous eyes (Figure 1B), provides a possible adaptive explanation for why smaller wild guppies were also less likely to darken their eyes when encountering predators in our first experiment (Figure 1E). This body size effect, in addition to the possession of ornamental eye-sized black spots (Figure 1B), which would likely dilute any divertive effect of conspicuous eye colour, offers a likely explanation for why males, which are similarly sized to the non-benefiting small females, were also less likely to display black eyes. Larger body size is intrinsically linked with fecundity, with important consequences for a wide range of biological phenomena such as life history evolution [39] and sexual selection [40]. Whilst our study represents a single (albeit important) predator-prey interaction context, we note that guppies and other Poeciliids display particularly striking female-biased sexual size dimorphism [41]. In addition, a previous study on mustelids suggested that size-dependent benefits of divertive colour patterns may explain the presence of black tail tips in larger species such as stoats (*Mustela erminea*) but not weasels (*Mustela nivalis*) [42]. Whether divertive antipredator tactics such as deflection and the matador strategy we detail here contribute to the attainment of otherwise larger adult body sizes by ameliorating the costs of reduced agility in other taxa provide exciting avenues for future study.

A wide range of species have capitalised on the inherent conspicuousness of the vertebrate eye by incorporating eye-mimicking colour patterns on their body, with multiple studies showing these provide a range of antipredator benefits [6,7]. However, whilst the striking eye colouration of many frogs has been hypothesised to deter predators [43], we are not aware of any previous empirical studies demonstrating that the colouration of real eyes can be exploited as part of an antipredator strategy. The complexity of the antipredator strategy we detail here, where a colour pattern must be married with a precisely-executed escape response, lies in stark contrast to our current understanding of divertive antipredator tactics. These tend to be viewed as 'deflective' and are adaptive because a predator makes physical contact with a non-vital body part [6,11]. Whilst the hundreds of fish species that display eye-mimicking spots on their body are assumed to benefit from deflection, there have been few empirical studies directly showing that prey of any taxa do indeed escape attacks this way, despite convincing evidence that predator attacks are diverted by conspicuous colour patterns (e.g. [11]). Indeed, the costs incurred by predators causing damage to non-vital body parts [44] might make 'matador-like' mechanisms that involve the use of conspicuous body parts to misdirect predator attacks similar to what we report here, relatively widespread in agile prey with sensory and locomotory abilities that allow them to react sufficiently quickly to a predator's attack to escape unscathed.

In summary, this study introduces a previously undescribed antipredator divertive strategy that has implications for the evolution of body size, advances our understanding about the function of salient eyes, and details a methodological approach offering significant flexibility for studying the adaptive function of antipredator traits in species attacked by ballistic-hunting predators.

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Author contributions

DPC and SKD conceived the overarching programme of research. RJPH, DPC and SKD conceived and developed the hypotheses and designed the research. RJPH and JT compiled the Crenicichla visual system and developed the visually calibrated guppy and cichlid models in discussion with DPC and SKD. JT and SKD provided materials and RJPH and JT designed the tracking and predation-simulation software in discussion with DPC. RJPH coordinated experiments. RJPH, AMB, PRL, and LCN- J collected the data. JW provided videos for the live predation experiment, and IR provided materials and infrastructure in Trinidad. RJPH analysed the data in discussion with DPC, JT, SKD and JW. RJPH wrote the first drafts of the manuscript with DPC and JT and all authors contributed to the final version.

Declaration of Interests

The authors declare no competing interests.

Figure Legends

Figure 1. Predictors of eye colour expression in Trinidadian guppies.

(A) Example images of female (top two images) and male (bottom two) guppies displaying either black or silver irises (also see Video S1). (B) Predicted visual resolution of female guppy with different eye colouration at different distances based on pike cichlid visual acuity and colour vision. Rows of images from top to bottom refer to the original image, mean, mean + standard deviation, and maximum distance respectively that pike cichlids attacked guppies from based on our predation simulation experiment (see Figure 3). (C) Images of a live pike cichlid (left) and its associated biomimetic model (right). (D) Difference in probability of expressing black irises between inspecting and non-inspecting fish across all populations and stimuli. (E) Difference in probability of expressing black irises in inspecting female guppies depending on predator stimulus and predation environment. (F) Effect of body size and predation environment on probability of expressing black irises in female guppies when they encounter a predator. Error bars and shading denote the 95% confidence interval. See Table S1 for summary statistics.

Figure 2. Eye colour of biomimetic robots diverts attacks by predatory pike cichlids.

(A) Experimental setup for testing effect of guppy eye colour on aiming point of cichlid attacks (not to scale). (B) Aiming point (dotted line) of cichlids on robotic guppies was determined at the pre-acceleration (left) and the pre-contact phases (right) of the attack (see Video S1). (C) Eye colour of robotic guppies diverts attacks by pike cichlids (plotted raw data for pre-contact phase shown). X-axis denotes the proportion along the body length of the robot. Violin plots show median and interquartile range. See Table S2 for summary statistics.

Figure 3: The divertive effect of conspicuous eyes enhances the ability of black-eyed guppies to escape from pike cichlid attacks.

(A) Schematic adapted from actual trial of a live pike cichlid attacking a live black-eyed guppy separated by a transparent barrier during the simulation experiment. The cichlid's ballistic attack trajectories are shown based on its predicted aiming point depending on whether a guppy was expressing black (head-directed) or silver (centre-of-mass directed) iris colouration. Each frame of the guppy's escape sequence was scored as a 'capture' or 'escape' based on whether the shaded area overlapped the guppy's centre of mass (marked with an "X"; see Video S1). Guppy images denote escape trajectory at 0, 4, 10, 25, and 40ms intervals where 0 is the frame preceding the first movement of the fast-start. (B) Escape probability depends on whether the real eye colour expressed by a guppy matches the simulated attack point (determined from the robotic guppy experiment). (C) The relative benefit of eye colour depends on guppy body size. (D-E) Black-eyed guppies were slightly slower (moved a shorter distance) over the critical 30ms of the fast-start escape response but escaped at more tangential angles relative to the cichlids actual attack angle (also see Figure S1). Error bars and shading denote the 95% confidence intervals. See Table S3, S4 and S5 for summary statistics.

Figure 4. **Guppy survival during real predatory interactions is predicted by its escape trajectory in relation to an attacking cichlid's aiming point.** (A) Image sequence at 5ms intervals of pike cichlid attacking guppy. The red cross denotes the aiming point (here, towards the guppy's head) of the cichlid. (B) Interaction plot showing how guppy survival depends on its escape angle in relation to the aiming point of cichlid at the pre-acceleration phase of an attack. Videos reanalysed from [34] (see Video S1). See Table S6 for summary statistics.

STAR METHODS

RESOURCE AVAILABILITY

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Robert Heathcote (r.j.p.heathcote@gmail.com). This study did not generate unique reagents.

DATA AND CODE AVAILABILITY

Data used in this study are publically available at Mendeley Data; DOI: 10.17632/knxpdt8stw.1

EXPERIMENT MODEL AND SUBJECT DETAILS

This study used three species of fish: Trinidadian guppies (*Poecilia reticulata*) (n=364; 125 males and 239 females), Trinidadian pike cichlids (*Crenicichla frenata*) (n=52), and spangled pike cichlids (*Crenicichla alta*) (n=12). Guppies and Trinidadian pike cichlids were captured from the wild in the Northern Range mountains in Trinidad, Trinidad and Tobago. After capture fish were maintained in aquaria at University of the West Indies, St Augustine Campus. Spangled pike cichlids were F1 generation fish captive bred at University of Exeter from subadults originally imported from Venezuela.

METHOD DETAILS

Study species

Guppies (*Poecilia reticulata*) are small (10-40 mm body length), neotropical live-bearing fish with a native range that extends across Trinidad and Tobago and northern Venezuela. Trinidadian guppies have been used as a model system in evolutionary biology since they inhabit multiple river basins in the Northern Range mountains of Trinidad that differ repeatedly in predation pressure [22]. Specifically, waterfalls act as selective barriers to the guppies' major predators; piscivorous fish such as the pike cichlid (*Crenicichla frenata*), two-spot sardine (*Astyanax bimaculatus*), and wolf fish (*Hoplias malabaricus*) [22]. The stark difference in predation risk across the barrier waterfalls has led to extensive divergence in a suite of phenotypic traits such as morphology, colouration and antipredator behaviours, thus making this species an ideal system for studying how populations evolve in response to predation risk [22]. Pike cichlids in particular rely heavily on guppies as a major constituent of their diet [45], and are known to generate strong selection pressures on a wide range of phenotypic and life-history traits in guppies [39].

Experiment 1: Behavioural observations of eye colour during predator inspections

To determine the specific contexts in which different eye colours are expressed by guppies, we staged a series of behavioural trials where individual guppies from four different populations that varied in evolutionary history of predation risk were presented with differing degrees of threatening stimuli.

Fish capture and maintenance

Between February 2nd and April 14th 2016 we collected 250 guppies (125 of each sex) using seine nets from a high and low-predation population within each of the Aripo and Guanapo river basins in the Northern Range mountains in Trinidad (population details in [14]). High and low-predation populations (native predation risk) from each were categorised based on whether they contained their three major predators: pike cichlids, wolf fish and two-spot sardines [22].

Captured fish were transferred the same day to an indoor aquarium (160 x 50 x 50 cm (length x width x height)), with each population kept in separate aquaria, at the St Augustine campus of the University of the West Indies, Trinidad and Tobago. Each aquarium was provided with gravel and large rocks collected from the source river population to provide refuges and areas of feeding and enrichment. Fish were allowed to settle for at least three days whilst being fed *ad-libitum* with commercially available fish flakes (Tetramin Tropical Fish flakes, Tetra™). Water temperature in all aquaria was maintained at 24°C with a 12:12hr day:night cycle.

Creation of predator models

We used two types of predator stimulus in our experiment: visually-realistic models of pike cichlids, and a novel control stimulus (blue Playmobil™ brick). To create the model pike cichlids, we first made a realistic three-dimensional polyurethane 'fast-cast' resin cast of a cichlid made from an RTV silicone mould (Maragon, Hatfield, UK). This process allowed us to create highly detailed models where individual scalation and lateral line patterns are faithfully replicated. The silicone mould of the pike cichlid was made from an adult male pike cichlid (*Crenicichla alta*), a close and nearly indistinguishable relative of *Crenicichlia frenata* and a major predator of guppies in Venezuela, that was maintained in the lab in Exeter and was originally imported from Venezuela in 2015 as a subadult. We euthanised the pike cichlid using MS222 (Sigma Aldrich), and death was confirmed by pithing.

Visual calibration of model fish colouration

We made a total of three pike cichlid casts. To make the casts visually-realistic to guppy vision, we printed calibrated images based on a visual model-transformation of digital photographs taken of the lateral view of the same pike cichlid used to make the silicone mould, and adhered these to the cichlid casts. As we have previously discussed [14], guppies exhibit large degrees of inter-population variation in their colour vision due to the plasticity in opsin-gene expression driven by differing lighting environments experienced during ontogeny [27]. As this first experiment involved multiple guppy populations, we accommodated for this inter-population variation by using a visual-model of our calibrated camera, which displays broad spectral peaks that encompass the interpopulation variation of the guppies (see [46]).

We photographed a live and calm pike cichlid within a water-filled UV-transmitting PVC box using a colour-calibrated camera (Samsung NX1000 with Nikkor EL 80 mm lens; the camera was converted to full-spectrum and a Baader UV/IR cut filter was used to photograph in the human-visible range). Two grey standards made from sintered PTFE (Zenith 5% and 95%) were included in each photograph to control for lighting conditions and convert the image to linear, normalised reflectance [46]. Our camera and printer (a Hewlett Packard M551 laser printer fitted with standard HP 507 ink cartridges) were able to produce calibrated colours in the human-visible range (400-700 nm, see [46] for spectral sensitivity curves). Our system was colour-calibrated by printing a calibration sheet onto the same type of PVC film used to colour the fish models. The calibration sheet contained 1,026 squares, each having a colour coded by a randomly generated RGB value. The printed film was placed onto a flat surface spray-painted with White Plastic Paint (PlastiKote, Wolvega). The calibration sheet was photographed using the same calibrated camera and grey reflectance standard used for photographing the fish. Next, the normalised, linear pixel values measured by the camera for each of the 1,026 squares were calculated using the micaToolbox for ImageJ [46,47]. The RGB value of each square was converted from sRGB space (8 bits per channel) to linear RGB values. Finally, a polynomial model was fitted for each camera channel between the camera-measured and known linear RGB values. These polynomial models are similar to the methods used to convert between calibrated camera images and animal cone-catch quanta (see [46]). Once the polynomial models were generated, these were used to create colour-calibrated linear RGB images (in 32-bits per channel) from the calibrated fish photographs (also 32-bits per channel). These linear RGB values were then converted back to sRGB-space (8 bits per channel). The image calibration processing was all performed using custom-written code in ImageJ (available as a plugin on request). Finally, the colour-calibrated images were size-matched to the body size of the polyurethane cast cichlid and then printed onto the PVC film. We then spray-painted the three resin casts with the same spray paint used to calibrate the models (White Plastic Paint (PlastiKote, Wolvega)) so that the same background colouration was used for calibration and creating the models. After spraying the casts, we wrapped them in the calibrated PVC film (with the printed-side against the model), ensuring that the lateral images deformed flexibly around the fish to ensure the fish colouration and patterning matched its appropriate anatomy (Figure 1B). The wet spray paint meant that the PVC film strongly adhered to the models once it had set, forming a waterproof 'skin'. Before trials commenced, the models were submerged in aged (de-chlorinated) tap water for 24 hours before experiments started. All guppies during the study displayed characteristic inspection behaviours once they came within 30 cm of the predator stimulus, including a clear avoidance of the 'attack cone' towards the head [48], indicating they recognised the models as a piscivorous fish.

Predator inspection trials

We carried out the behavioural predator inspection trials in aquaria measuring 90 cm x 30 cm (length x width) with a water depth of 10 cm. Prior to trials beginning, a single guppy was released into a compartment at the end of the experimental aquarium (the end 15 cm of the aquarium) which had a remotely operated opaque door allowing access to the rest of the tank, and contained a refuge (small plastic aquarium plant and acetate container). Guppies were transferred into this compartment using a soft aquarium net and left to habituate for two minutes before the compartment door was remotely raised (gently so as not to startle the guppy), after which the guppy could exit and explore the entire aquarium ('exploration phase'). After five minutes had elapsed, the guppy was then gently coaxed back into the end compartment using an aquarium net, and the remote opaque door was lowered. If the guppy showed any antipredator responses (e.g. fast-start or freezing behaviour) in response to being coaxed into the compartment, or when the remote door was operated, then the trial was terminated and restarted with a new fish. Whilst the guppy was back in the refuge compartment, the predator stimulus was then added to the opposite end of the tank without disturbing the guppy. Predator stimuli were suspended 2 cm off the bottom of the tank, 10cm from the far wall at the opposite end of the aquarium at a 45° angle facing the compartment door, ensuring that the full lateral profile of the cichlid model was visible to the guppy (to enhance probability of its recognition as a predator) whilst also encouraging inspection behaviour since the guppy could still approach whilst avoiding the cichlid model's 'attack cone' [48] (the same protocol was taken with the Playmobil brick to ensure consistency in presentation). After two minutes had elapsed and the predator stimuli had been positioned, the compartment door was again remotely raised and the guppy allowed to exit the compartment and interact with the predator stimulus ('test phase'). During both the exploration and test phases of the trials, we constantly recorded the eye colour of the focal guppy, as well as its position within the aquarium. Determining a guppy's relative position within the test aquarium was facilitated by dividing all the aquaria into eight equally-spaced zones and recording which one the guppy was in at any one time. Trials were scored live by an observer who was blind to the population origin of each guppy being tested, and who scored the trials in a manner that had no noticeable effects on guppy behaviour. After five minutes had elapsed, each guppy was removed from the aquaria and photographed against a 5 cm ruler. The standard body length (nose tip to end of caudal muscle) of each guppy was measured later in ImageJ from the photographs.

Modelling conspicuousness of eye colouration based on pike cichlid vision

We used the Quantitative Colour Pattern Analysis (QCPA) framework [49] to simulate a pike cichlid viewing a female guppy from three ecologically-relevant distances (Figure 1B). We analysed the same images of 10 female guppies used to create the black and silver-versions of our biomimetic robotic guppies (see methods below). Based on the average pike cichlid eye size and visual acuity of other south American species of cichlid, we assumed a visual acuity of 12cpd, and viewing distances relate to the minimum, mean and maximum attack distances that pike cichlids initiated their attacks from in our simulation experiment. Edges were reconstructed using the RNL ranked filter with a minimum of 5 pixels per minimum resolvable angle. Pike cichlid LWS, MWS and SWS are shown in Figure 1B in place of RGB respectively, following a square-root transformation.

Experiment 2: Predator responses to guppy eye colouration

To determine whether guppy eye colouration influences attacks by predators, between March and May 2018 we caught 52 wild pike cichlids from the Tacarigua river in the Northern Range mountains in Trinidad (GPS coordinates: 10°41.56' N 61°21.01' W). Cichlids were caught with a light-weight #3 fly fishing rod using a barbless hook baited with a dead female guppy. Female guppies were caught in the same river using a seine net and killed by a rapid strike to the head and immediately pithed. Pike cichlids were transferred the same day to large (1.5m x 1.5m) circular outdoor holding tanks placed outside in the shade at the University of the West Indies, St Augustine Campus. Holding tank temperature and light cycle followed ambient conditions (water temperature varying between 25°C and 27° and day:night cycle of approximately 12:12 hours). Pike cichlids were moved to their experimental aquaria (dimensions: 90 x 30 x 40cm (length x width x height)), to acclimatise for at least one week before trials started. Each experimental aquarium contained a 15cm long black PVC pipe at one end of the aquaria as a refuge for the cichlid, as well as freshwater plants collected from the streams where the pike cichlids were caught (Figure 2). A small air-fed canister filter was placed in the corner of the tank to maintain water quality. Pike cichlids were fed daily in these experimental aquaria as per their holding tanks.

Creating the robotic guppy system

Using a similar protocol as used to create the model cichlids, we made 40 polyurethane model guppies (20 of both eye colours) to present to our pike cichlids in a robotic prey experiment. Since we found no predictors of eye colour variation in male guppies, we restricted the robotic guppy predation experiment to just females. Model guppy casts were made from 20 female guppies captured from the same high predation Aripo population used for Experiment 1 in February 2017. Each female was euthanised by anaesthesia in MS222 and then pithed to confirm death, before being used to make the silicone mould. Photographs used to create the realistic colour patterns, in this case calibrated to a pike cichlid visual system, were taken of adult lab-bred female descendants of a high-predation Aripo population from Trinidad (population coordinates: 10°39’031’N; 61°13’40’W) that were maintained in the lab in Exeter.

Pike cichlid visual system

We generated a *Crenicichla frenata* colour vision model based on peak spectral sensitivities determined for this species of 614nm (LWS), 547nm (MWS) and 480nm (SWS) (i.e. a trichromatic visual system with no UV sensitivity [50]), with spectral sensitivity curves fitted using the Govardovskii model [51]. Ocular transmission data are not available for this species and so we used the full transmission curves based on a closely related central American cichlid, the rainbow cichlid (*Herotilapia multispinosa*) [52]. To more closely match the Trinidadian pike cichlids, we shifted the curve 31nm to those of a sister species also within the *saxatilis* pike cichlid complex, *Crenicichla lepidota*, which has a 50% cut-off at 400nm [52]. The spectral sensitivity curves used are available in the micaToolbox [46].

Guppies were photographed using a calibrated camera with known spectral sensitivity curves (Samsung NX1000 converted to full-spectrum sensitivity with Nikkor EL 80mm lens and Baader UV/IR cut filter). The micaToolbox was used to generate pike cichlid cone-catch images of the guppies. Next, we used a printer calibration process which allows us to convert from pike cichlid trichromatic cone-catch images to calibrated printer output similar to our method for creating the model pike cichlids. As we have discussed elsewhere [14] this colour calibration process faithfully recreates colours for opaque objects, although we found that the slightly translucent bodies of real guppies resulted in their appearing brighter underwater than our models. To compensate for this, we therefore increased the brightness of the images until they matched that of live guppies that had been photographed underwater, ensuring that all images were increased by an identical level.

To create black- and silver-iris models, photographs were taken of each live guppy when they expressed silver-irises and subsequently when they expressed black-irises following induction of anaesthesia with MS222, which causes the body melanophores (including those in the iris) to disperse. Because black-iris expression during predator inspections is not associated with corresponding body melanophore expression (Video S1c) as they are when treated with MS222, we created our corresponding silver-iris and black-iris model treatments by using GNU Image manipulation program (GIMP) software to 'cut' out the black irises from the MS222 treated fish photograph, and superimposing these onto a photograph of the same fish that was expressing normal body pigmentation (i.e. had not been treated with MS222). To control for any effects that the photographic manipulation procedure may have had, we created the silver-iris models using exactly the same procedure but superimposing the eye from a different photograph of the same fish when it was still expressing silver-irises. Following their photographs, guppies were immediately euthanised with an overdose of MS222, with death confirmed by pithing, and these same fish were then used to create the silicone moulds to create the casts for the model guppies (so that the photograph and cast of each individual fish were matched when creating each model); see below.

Finally, the guppy images were size-matched to the polyurethane casts, and the top of the dorsal region was stretched using GIMP to allow the printed PVC 'skins' to conform over our three-dimensional models. Applying the skin to the model guppy was done in an identical manner to that pike cichlid models detailed above.

Robotic system

We next created an automated system that allowed our model guppies to ‘inspect’ the pike cichlids within their aquaria using a 4- phase, 1.8- degree stepper motor (PC Control Ltd, Kettering). The inspection rig was created by attaching a single guppy model to a transparent 90cm long loop of 7-lb monofilament fishing line, which was then wrapped around two 35mm pulleys that were themselves attached to a small 30mm diameter rubber aquarium suction cup to allow attachment to the aquarium wall. These were then placed at opposite ends of the experimental aquarium holding the pike cichlid. The pulley at the end furthest from the pike cichlid refuge was attached to the stepper motor shaft by another loop of monofilament line, which acted as the drive belt, allowing the guppy to travel from this position down the aquarium towards the pike cichlid as the stepper motor shaft rotated. We programmed the guppy’s movements to match the saltatory swimming behaviour of an inspecting guppy approaching a predator.

Trial protocol

Before undergoing a trial, each pike cichlid was food-restricted for 48 hours. The robotic guppy rig was installed directly into the pike cichlid’s aquarium, with the model guppy hidden behind a visual barrier at the opposite end of the aquarium to the pike cichlid’s refuge (Figure 2A). A high-speed camera (fps1000hd; Slow Motion Camera Company) recording at 1000Hz and a GoPro Hero 4 was mounted directly above the aquarium to record the attack behaviour and kinematics of the pike cichlid. The cichlid was then allowed to settle for one hour before the trial started. To start a trial, the stepper motor system was started remotely, out of view of the pike cichlid, so that the model guppy ‘swam’ down the aquarium towards the entrance of the pike cichlid’s refuge in a punctuate motion characteristic of inspecting fish. Trials were only started once the pike cichlid was within its refuge with its head facing outwards in a characteristic ambush posture. To avoid the possibility of the pike cichlid damaging its jaw by hitting the aquarium wall due to overshooting the model guppy during an attack, we also placed a strip of opaque polythene sheeting against the wall immediately behind the guppy model. This also stopped any reflections from the guppy appearing on the glass wall due to the glass-water interface.

After the model guppy had reached the end of the aquarium, the rig was reset, the guppy model was replaced with the alternative eye colour version from the same model pair, and the pike cichlid was again allowed to settle for an hour before the trial was rerun. Each pike cichlid was therefore sequentially presented with an otherwise identical pair of model guppies that differed in their eye colour. We alternated the presentation order of different eye colours from each model guppy pair to avoid any confounding priority effects of eye colour. After a pike cichlid was presented with two model guppies, it was immediately fed with a euthanised guppy (killed using a strike to the head and pithing), removed from its test aquarium, and placed into an outdoor water tank with identical maintenance conditions as described above. Cichlids were kept in this tank until they were released back into their original population in the Tacarigua river after the experiment had ended.

Video scoring

For each trial where the pike cichlid attacked a model guppy, we extracted all high-speed footage frames from the start of the 'S bend' attack until the point just prior to the pike cichlid making physical contact with the model guppy. Using these frames, we then determined a cichlid's aiming point on a model guppy's body at two phases of the attack: at the period during the S-bend immediately prior to acceleration, specifically the first frame where the pectoral fins (which are held perpendicular to the body prior to an attack) started to move against the body to help launch the cichlid forward ('pre-acceleration phase'), and also just prior to physical contact ('pre-contact phase') at the frame immediately prior to the point that the model guppy moved in response to the suction forces generated by the opercular expansion of the attacking cichlid. We quantified the aiming point on the model guppy's body as the position that was bisected by the line of symmetry projected from the pike cichlid's head (Figure 2A), and converted this into a percentage, where 0% = the tip of the model's nose and 100%= the end of the model's tail. Using the high-speed footage, we also calculated the distance between the pike cichlid and the model (from the end of the cichlids jaw to where the aiming point bisected the model), the angle of attack (i.e. the angle of the attack trajectory relative to the angle the guppy model was facing). All video scoring was done by a single observer (RJPH) who was blind to the eye colour of the robotic guppies in each video (eye colour in the models could only be viewed from a lateral viewpoint, and trials were given a random code name). We were not able to analyse the footage of five cichlid attacks due to a software fault with the high-speed camera.

Experiment 3: Simulation of predation outcomes based on guppy eye colour

Whilst we found that displaying black irises significantly diverts predator attacks towards a guppy's head, our robotic trials suggest that this divertive effect would still cause guppies to be eaten if they failed to react to an attacking cichlid. We therefore wanted to determine whether this divertive effect is actually adaptive when combined with a guppy's escape response. Ethical issues associated with staged laboratory trials on predation events between vertebrates have historically been contentious, particularly in Europe where live predation experiments are rarely carried out between vertebrates [33]. To overcome this, we took advantage of the extreme high-speed nature of cichlid-guppy predatory interactions to simulate predation outcomes based on real predator-prey interactions. In most predator-prey interactions between fish, particularly involving ambush predators such as pike cichlids, the refractory period of the fast-start associated neurons is longer than the average duration of a predator's lunging attack, creating a scenario where both the attack and ensuing escape trajectories are largely ballistic [30,35] and thus neither party responds to subsequent sensory information until the outcome (capture or evasion) of the interaction is determined (typically 10-30ms in cichlid-guppy interactions [34]).

We exploited the neural constraints associated with this phenomenon and staged interactions between real pike cichlids and guppies where both parties were separated by a transparent acetate barrier, allowing the guppy to respond to an attacking pike cichlid with a fast-start, but where the attack outcome (capture/evasion) was simulated. To further validate this method specifically for our system, we also reanalysed video footage from a previously published experiment on live predation trials between pike cichlids and guppies [34] (see Experiment 4). Consistent with pike cichlid attacks being ballistic, we found that the pre-acceleration phase aiming point of the cichlid on a guppy's body strongly predicted its pre-contact phase aiming point, where pre-contact phase aiming point was based on the guppy's position prior to the start of its fast-start escape.

Experimental animals and apparatus

We staged predator-prey interactions between trios of guppies (n=114) and individual pike cichlids (n=12) where both parties were separated from one another by a clear acetate barrier. Guppies were lab-bred fish originating from a high-predation region of the Aripo river, whereas the pike cichlids were first-generation lab-bred *C. alta* (see details in Experiment 1 for information about lab cichlids).

All trials were carried out in a test aquarium measuring 60 x 30 x 10cm (length x width x water depth) that was divided into two compartments by a sheet of transparent acetate (Figure 3A). Examining the test tank underwater with a GoPro Hero 4 camera recording at 4K resolution showed that the acetate divider was virtually invisible when viewed from the perspective of the pike cichlid. One hour before a trial started, a single pike cichlid was moved from its home tank within its shelter, a 15cm long black PVC pipe, and placed into one of the compartments where it was allowed to settle. After the hour, a trio of lab-reared female guppies originating from a high-predation region of the Aripo river in Trinidad were introduced to an opaque closed-off refuge to habituate for five minutes. After this habituation period, a remotely activated door was raised, allowing the guppy trio to exit their refuge and approach the pike cichlid to inspect it. Pike cichlid attacks on guppies were then recorded at 1000Hz with a dorsally mounted high-speed camera, and at 30Hz with a GoPro Hero 4 placed at the side of the aquarium to record the eye colour of the guppies at the point of their being attacked. After the trial, guppies were removed and their standard body length was measured to the closest mm. All guppies within a trio were individually identifiable due to being slightly different body sizes.

Video analysis

Only trials where the guppy began its fast-start response before the pike cichlid made contact with the acetate barrier were analysed, to ensure the guppy's escape trajectory was unaffected by any non-directional tactile stimulation caused by the acetate barrier vibrating in response to being hit by the cichlid. We therefore had 38 complete trials, during which we recorded 73 attacks on different guppies (no guppy was attacked more than once). We designated the attacked guppy (and therefore the one that was analysed in the footage) as the one that was clearly being aimed at by the cichlid. Pike cichlids visually track their prey before launching a ballistic attack, maintaining the point of aim within the centre of their binocular visual field throughout the S-bend strike (see Experiment 3 & 4; [34]). Our robotic experiment and reanalysis of the published live-predation experiment we describe below, show that the aiming point at the pre-acceleration phase strongly predicts the aiming point along the guppy's body at the pre-contact phase (the latter of which we could only rarely obtain from our trials since the transparent barrier stopped the cichlids getting close enough to the guppy). We therefore used the pre-acceleration phase aiming point of the pike cichlid to project its attack trajectory towards a guppy to calculate simulated survival probabilities based on different eye colours. From the high-speed footage, we isolated 30 sequential frames (i.e. over a duration of 30ms) starting from the frame immediately prior to the first recorded movement of the guppy's fast-start in response to being attacked by a cichlid. We chose this time period because it encompasses the interval during which the majority of guppies have been previously shown to be captured during live predation trials [34].

Using an automated custom-written plugin with ImageJ (available upon request), we then calculated the minimum distance between the attack trajectory and the guppy's centre of mass for every frame of the fast-start sequence. Our plugin used a convolution-based approach, which detected the centre and orientation of an ellipse matching the dimensions of the guppy's abdomen for each frame (the location and length of the guppy's abdomen was specified at the beginning of the tracking sequence). We equated the central point of the ellipse as the guppy's centre of mass (approximately 35% down the guppy's body) after verifying that it was the body region that moved the least as the guppy rotated during its fast-start, following other studies [34,53]. At the beginning of the video sequence, we also extended two straight lines from a point at the centre of the pike cichlid's head (equidistance between the cichlids eyes) until it bisected the guppy's body at either 18% or 43% down its body length, corresponding to the bisection point determined from our robotic trials for either a black-iris or silver iris fish respectively. The bisection points were assigned by a single researcher (RJPH) who was blind to the eye colour being expressed by the guppy being analysed since eye colour is only visible from a lateral viewpoint. Our ImageJ program then automatically recorded the x-y coordinates and orientation of the guppy, as well as measuring the shortest distance between the two attack trajectories and the guppy's centre of mass, for every frame of the fast-start sequence (Figure 3A).

In predatory fish that combine rapid whole-body lunges ('rams') with suction-generating opercular expansions, the 'degree of focus' of water that enters the mouth is particularly high during suction (i.e. it is mostly the water immediately in front of the mouth that is engulfed along with the prey item) [54]. Because a prey's centre of mass is particularly susceptible to the propulsive forces generated by the opercular expansion from suction feeding predators such as pike cichlids, we assumed that a predator's gape must therefore overlap the guppy's centre-of-mass for it to be captured, similar to other studies [29]. We therefore categorised each frame of the fast-start as a 'capture' or 'escape' depending on whether the distance between the attack trajectory and the guppy's centre of mass was lower or higher, respectively, than half the width of the pike cichlid's gape. The cichlid's gape width was measured directly from the attack footage as the lateral transverse distance at the base of the jaws, which corresponds to the maximum width the mouth can open. This categorisation was then used as the binomial response variable in our analysis to determine whether escape probability was influenced by a guppy's eye colour.

Finally, we also measured additional parameters that relate to fast-start performance in guppies that are known to predict survival probability during encounters with predators [34] and compared these between individuals expressing different eye colours. Specifically, we measured the distance a guppy travelled for the full 30ms of the fast-start, its starting angle relative to the pike cichlid's (where 0° was where the guppy and pike cichlid directly faced one another and 180 was equivalent to the guppy facing in the same direction as the cichlid; guppy heading was determined by the orientation created by a straight line of symmetry running from its centre of mass to the tip of its nose, whereas the pike cichlid heading was determined by a line of symmetry projected anteriorly from the centre of its head), and finally the escape trajectory of the guppy, determined by the angle created by its change in heading between its first and 30th frame of the fast-start.

Experiment 4: Validation analysis based on previously published experiment on live predation trials

As further validation of our experimental methods, we took advantage of existing video footage from a live predation experiment between Trinidadian pike cichlids (*Crenicichla frenata*) and guppies that was used in a previously published study [34]. Specific details of the methodology are provided in the original study [34], but briefly we reanalysed videos involving 53 guppies presented individually to a solitarily-housed pike cichlid, and the resulting predatory interactions filmed using a high-speed camera with a frame rate of 1000Hz. Guppies were lab-reared individuals that originated from wild stock from five streams in Trinidad or from pet-shop bought domesticated strains. All predator-prey interactions took place in aquaria with a transparent bottom and were filmed from below whilst being dorsally illuminated. The cichlid was provided with a single shelter within which it would wait before ambushing an introduced guppy. Guppy eye colour was not recorded during these trials, and, in contrast to our study, the cichlid was often cryptic and many attacks were on non-inspecting guppies.

We re-analysed the footage from this study for two reasons. Firstly, we wanted to determine whether pike cichlid attacks are ballistic and thus whether the pre-acceleration aiming point of a pike cichlid was a reliable proxy for determining its attack trajectory, and thus whether it could be used for calculating the simulated escape probabilities in Experiment 3. Secondly, we wanted to verify whether a guppy's survival was influenced by its escape trajectory in relation to where a pike cichlid was aiming during the pre-acceleration phase of its attack, confirming that diverting a predator's attack has the potential for fitness benefits.

For each trial, we measured five variables for our primary analyses: aim during pre-acceleration attack phase, aim during pre-contact phase, guppy escape trajectory, guppy-cichlid starting angle, and whether a guppy was eaten or not. We recorded the pre-acceleration aiming point of the cichlid in an identical manner as our robotic experiment described above. To make the aiming point during the pre-contact phase comparable between trials where the guppy escaped and where it was eaten, we used the guppy's position at the frame immediately prior to the first noticeable movement of its fast-start as our reference for calculating the aiming point, and the point the aim was measured was when the cichlid was within 25% of the guppy's body length of this position (so that the aiming point was taken before the guppy would have started to become engulfed by the cichlid). The guppy escape trajectory was taken as the angle created by the guppy's centre of mass for the first 30ms of the fast-start, where the start was the frame immediately preceding any noticeable movement of the guppy. The guppy-cichlid starting angle was calculated in an identical manner to the method we explain for the simulation trials, where 0° denoted as when the guppy and cichlid were directly facing one another.

All variables were scored by a single observer (RJPH), who was blind to the outcome of the trial on whether the guppy was eaten or not; trials were blinded by a scientist not involved in the study (Fiona Moultrie, University of Oxford; see acknowledgements) who removed the final frames of the video sequence that showed the predation outcome, and assigned a coded name for each trial which was then revealed after the videos had been scored. After the trials were analysed, we also measured the minimum distance a guppy's centre-of-mass came within the furthest edge of a pike cichlid's gape during a fast-start for those interactions where a guppy escaped. The purpose of these final measurements was to qualitatively assess the margins of error that determine a capture or escape during a pike cichlid-guppy interaction.

We excluded 11 trials from our analysis due to the guppy being within a single body length of the aquarium wall (and thus the rotational aspect of its fast-start would have been compromised), if the pike cichlid's head was not visible at the start of its attack, or if the guppy being attacked was positioned at such an angle to the cichlid's shelter that required the cichlid to bend as it exited the shelter.

Ethics statement

The robotic guppy experiment in this study was approved by the Psychology Ethical Review Group at the University of Exeter, and the anaesthesia and predator inspection trials were carried out under a UK Home Office Project License (number 30/3308).

Quantification and statistical analysis

Experiment 1

In all analyses on eye colour variance, we constructed generalised linear mixed-effects models (GLMMs) with Bernoulli outcome binomial error structures, where an incidence of black iris was coded as 1 and silver iris coded as 0. The significance of model terms was determined using likelihood-ratio tests between nested models with and without the factor of interest. We first tested whether black iris expression was predominantly linked to predator inspection in our trials. We classified guppies as inspecting once they came within 30 cm of the predator stimulus, due to this being the mean distance that wild guppies are known to initiate inspections from [45], and also during pilot trials was the distance that guppies would start to initiate the stereotypical saltatory movement patterns characterised by this behaviour [48]. We first ran a GLMM that determined whether fish were more likely to express black irises when they were inspecting (i.e. within 30 cm of the predator stimulus) compared to the time period they were not inspecting. Our model included inspection as a two-level factor, time spent inspecting in seconds as a covariate, and we also included the random intercept terms river basin, fish ID (to control for pseudoreplication since each fish was represented twice) and observer ID. After finding that guppies were substantially more likely to display black irises during predator inspections (see main text and Figure 1), we wanted to identify how eye colour varied under different contexts during the inspections themselves. Due to issues of model convergence, we ran a GLMM on each sex separately, and included the native predation risk level (high or low), the predator stimulus (model pike cichlid or blue Playmobil brick), the guppy’s standard body size in mm as a covariate, and the interaction between these three variables as the predictors in our analysis. We also included river basin and cichlid model ID as random intercept terms. We found no significant effect of any of these predictors on the incidence of black iris expression in male guppies (Table S1). For female guppies, the two-way interactions between native predation risk and body size, as well as the interaction between native predation risk and predator stimulus treatment, significantly predicted differences in eye colour expression during inspections (see main text, Figure 1, and Table S1).

Experiment 2

In 10 trials the pike cichlid attacked the model multiple times. These trials were scored and analysed, with pseudoreplication being controlled for statistically using an appropriate random effect structure (see below). We ran two linear mixed models with pre-acceleration or pre-contact aiming point as the two separate response variables. In both models, we included eye colour, angle of attack and trial number as fixed effects, and model ID, cichlid ID, and trial ID as random intercept terms. For both pre-acceleration and pre-contact phases of the attack, there was no effect of attack angle or model presentation order, but black-eyed robots were attacked significantly anteriorly compared to silver-eyed robots (Table S2; Figure 2C).

Experiment 3

To determine whether eye colour influences escape probability, we constructed a GLMM, with escape probability as the binomial response variable (coded as either a 1 (escaped) or a 0 (caught)). Our model predictors were eye colour (silver or black) expressed by the guppy at the time it was attacked, ‘simulated eye colour’ (silver or black) based on where the attack trajectory bisected the guppy, guppy length (mm), and the interaction between these three variables. We also included trial ID as a random effect, and to control for the replicated observations for each individual guppy, and the replicated use of the 12 pike cichlids across different trials, we also included the pike cichlid and guppy’s unique ID as random intercept terms. Finally, we included the quadratic transformation of 'frame number' (1:30) nested within guppy ID as a random slope term to control for the autocorrelated structure of these data.

After finding a significant effect of the three-way interaction (Binomial GLMM: χ^2=6.37, P=0.012, Table S3), we calculated the estimated marginal means (least-squares means) using the *emmeans* package in R to directly compare guppy escape probabilities across different contrasts of the interaction, using a Tukey's correction for multiple testing to assess significance for each contrast. To assess the overall benefit of eye colour across all body sizes, we held body size at its mean level (21.9mm) when directly comparing escape probabilities between different contrasts of real and simulated eye colour (Table S4), whereas we also compared the different slopes between escape probability and body size to assess how the slopes differ across different eye colours (Table S4). To compare the different kinematic properties we recorded for each guppy, we ran three linear mixed-effects models (LMMs) with distance travelled, escape trajectory, and maximum acceleration over the course of the fast-start. Escape trajectory was calculated as the change in angle created from the guppy's heading at its start point (with heading defined as the angle created by a line of symmetry drawn from its centre of mass to its head) to the heading at the 30th frame of its fast-start. Maximum acceleration was taken as the maximum positive change in speed for a sequential series of 5ms intervals over the course of the 30ms fast-start interval. For all models, we included guppy-cichlid relative starting angle, as well as the cichlid ID and trial ID as random effects. In all three models, we included eye colour and the quadratic function of guppy-cichlid relative starting angle and their interaction as fixed effects, as well as the guppy's length and the guppy-cichlid distance (in mm) as covariates. We found that 28 out of the 73 fast-starts (38%) were actually directed towards the pike cichlid, similar to other studies suggesting that fast-starting fish occasionally utilise a 'protean' escape strategy [55]. Because a towards response is likely to require a different escape trajectory compared to an away response for the divertive strategy we report here to be beneficial, we included this variable as a two-level factor interacting with guppy-cichlid relative starting angle and guppy eye colour in our escape trajectory model.

Black-eyed fish were slightly (but statistically-significantly) slower than silver-eyed fish (Figure 3D). As might be expected, guppies were faster when they were closer to a pike cichlid before an attack was initiated (Figure S1). There was a significant effect of guppy-cichlid starting angle on the distance covered by guppies during the fast-start, with the slowest speeds being realised when the guppy was facing approximately 40-100° in relation to the cichlid (Figure S1), possibly due to this also being the relative angle where black-eyed guppies escaped at the strongest tangential angles, which may have come at the cost of slower speed (see main text; Figure 3D & Figure 3E; [29]). We found no significant predictors for maximum acceleration. In our model on escape trajectory, after finding a significant interaction between escape direction, guppy-cichlid relative starting angle and eye colour (Table S5), we directly compared the different level contrasts of the factors of this interaction using estimated marginal means with a Tukey's correction for multiple comparisons (and a Kenward-Roger method for calculating degrees of freedom), holding the guppy-cichlid starting angle at its mean of 57° for each contrast. We found that silver-eyed fish had a substantially lower escape trajectory compared to the black-eyed guppies when escaping away from the cichlid (105.6° ± 16.9° for black-eyed fish versus 48.6° ± 12.2° for silver-eyed fish (mean ± SE); t=2.76, P=0.038), but the trajectories did not differ between eye colours when the fish were escaping towards the cichlid (57.9° ± 25.4° for black-eyed fish versus 100.5° ± 19.6° for silver-eyed fish (mean ± SE); t=1.33, P=0.548); Figure 3E)

Experiment 4

Analysis and results

We first ran a Pearson's correlation test to determine whether pre-acceleration aiming point is correlated with the pre-contact aiming point in all trials, after first confirming the test assumptions were met. We found that, indeed, both were highly correlated (Pearson's correlation test: $t = 4.49$, $df = 37$, $P<0.001$, coefficient=0.59). To confirm that this result was not simply because the pike cichlids were catching guppies before they had had a chance to move out the way, and thus the cichlid did not need to alter its trajectory, we re-ran the analysis on only those trials where the guppy escaped, again finding a strong correlation, and thus validating our use of the pre-acceleration aiming point as a proxy for the attack trajectory in our simulation trials (Pearson's correlation test: $t= 4.18$, $df = 26$, $P<0.001$, coefficient=0.64).

To determine whether a guppy's chance of escaping a cichlid attack is influenced by how its escape trajectory interacts with the predator's aiming point, we ran a generalised linear model where the predation outcome (1= guppy eaten, 0= guppy escaped) was used as the binomial outcome. We included pre-acceleration aiming point, guppy escape trajectory, guppy cichlid starting angle, and an interaction between these three variables. Our final model showed a strong two-way interaction between the pre-acceleration aiming point and the guppy's escape trajectory on its probability of surviving: (binomial GLM: $\chi^2=22.69$, $P<0.001$, Table S6, Figure 4).

In 15 out of 37 (40%) unsuccessful attacks, an escaping guppy came within a single body length of the pike cichlid's gape, illustrating the small margins that determine a guppy's survival or death during an attack from a pike cichlid, and how traits that create even a slight diversion of the predator's attack path may create opportunities for selection to arise.

Overall these trials validate and support our experimental protocols. A cichlid's probability of catching a guppy depends strongly on its aiming point on the guppy's body in relation to the escape trajectory of the guppy. Guppies attacked towards their head which subsequently escape at a strong angle to their original heading are highly likely to avoid being caught. Concordantly, our experiment trials show that black-eyed fish are more likely to be attacked towards their head, and are also more likely to execute an escape trajectory tangential to their original heading, explaining their higher simulated escape probability.

Legends for Supplementary Videos

Video S1. Example video sequences of the observations and experimental protocols used in this study. Related to Figures 1-4. (a & b) Underwater footage of wild guppies in a Trinidad stream displaying black eye colouration in the presence of a predatory acara cichlid (*Aequedens pulcher*) (a) or a model pike cichlid (*Crenicichla frenata*) used in this study (b). (c) Footage showing the eye colour change of a female guppy (leading guppy in clip) from silver irises to black irises when it inspects a live pike cichlid in its refuge. (d) Example high-speed footage of a pike cichlid attacking a biomimetic robotic guppy that was used to determine how eye colour diverts the aiming point of predator attacks. (e) Example high-speed footage of our simulation method of a real cichlid attacking a live guppy, where both are separated by a transparent acetate barrier. The guppy initiates its fast-start before the cichlid makes contact with the barrier, allowing the calculation of minimum distances between the cichlid's attack trajectory and the guppy's centre-of-mass (yellow lines) created at each frame of the video. In this case, we simulate the escape probability of a guppy displaying black irises. (f) Example video reanalysed from [34] used to confirm the ballistic interactions of cichlid attacks on fast-starting guppies.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Trinidadian guppy (*Poecilia reticulata*).	Northern Range mountains, Trinidad and Tobago.	NA
Trinidadian pike cichlid (*Crenicichla frenata*)	Northern Range mountains, Trinidad and Tobago.	NA
Spangled pike cichlid (*Crenicichla alta*)	Captive bred, University of Exeter, UK	NA

Figure 1

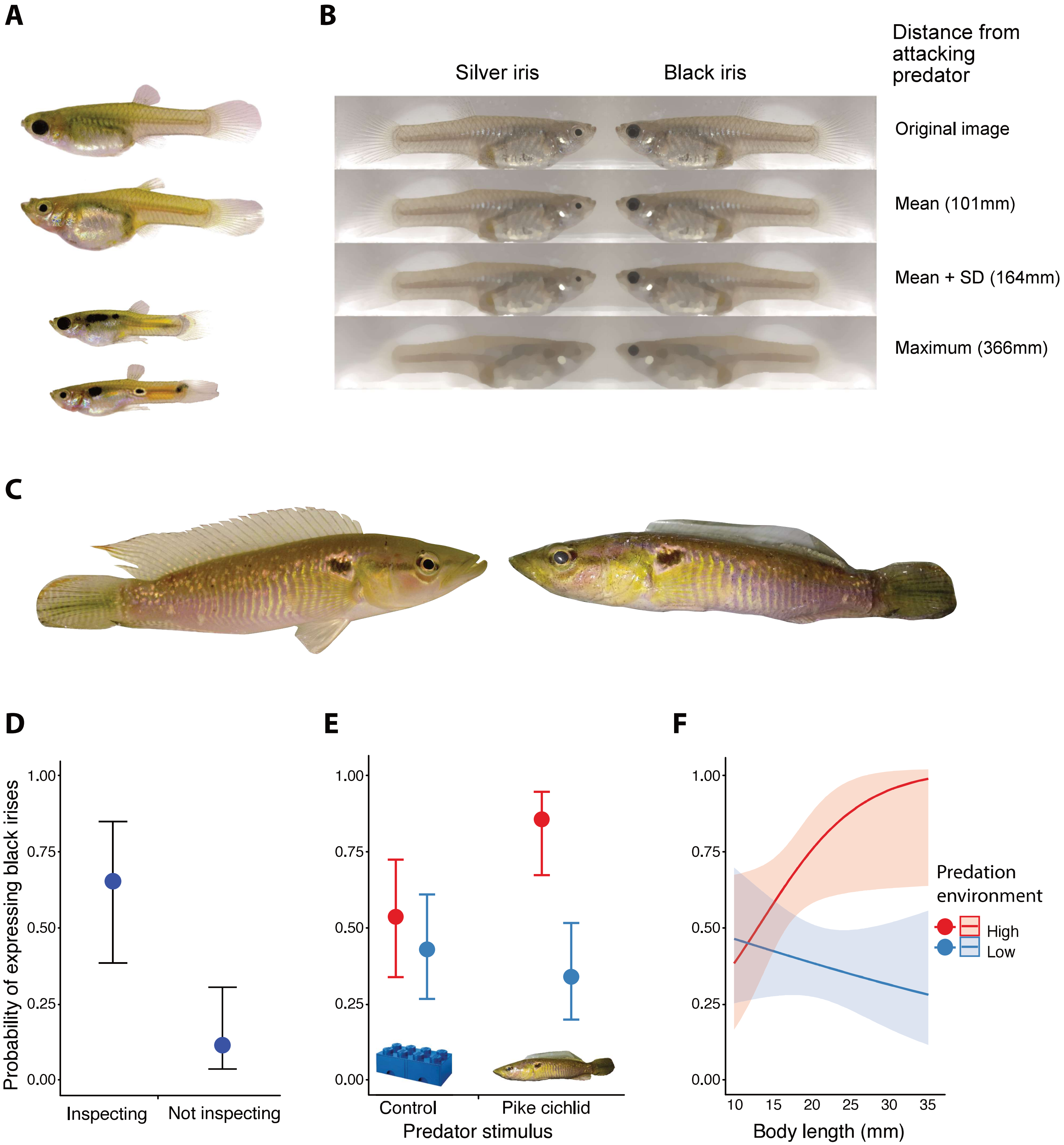

A
B
Silver iris
Black iris
Distance from attacking predator
Original image
Mean (101mm)
Mean + SD (164mm)
Maximum (366mm)
C
D
Probability of expressing black irises
1.00
0.75
0.50
0.25
0.00
Inspecting
Not inspecting
E
1.00
0.75
0.50
0.25
0.00
Control
Pike cichlid
Predator stimulus
F
1.00
0.75
0.50
0.25
0.00
10
15
20
25
30
35
Body length (mm)
Predation environment
High
Low

Figure 2

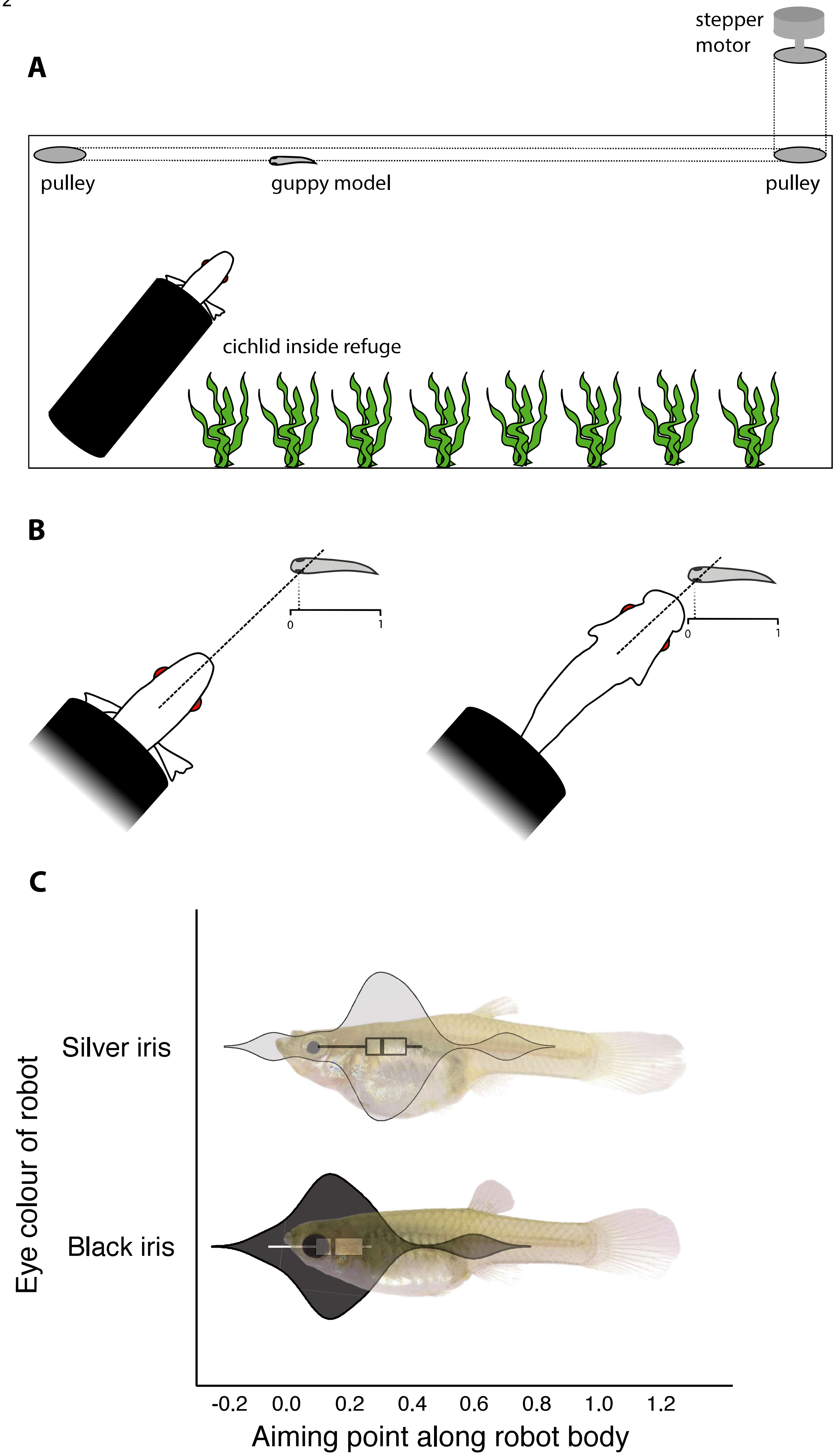

A
stepper motor
pulley
guppy model
pulley
cichlid inside refuge
B
0
1
0
1
C
Silver iris
Black iris
Eye colour of robot
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Aiming point along robot body

Figure 3

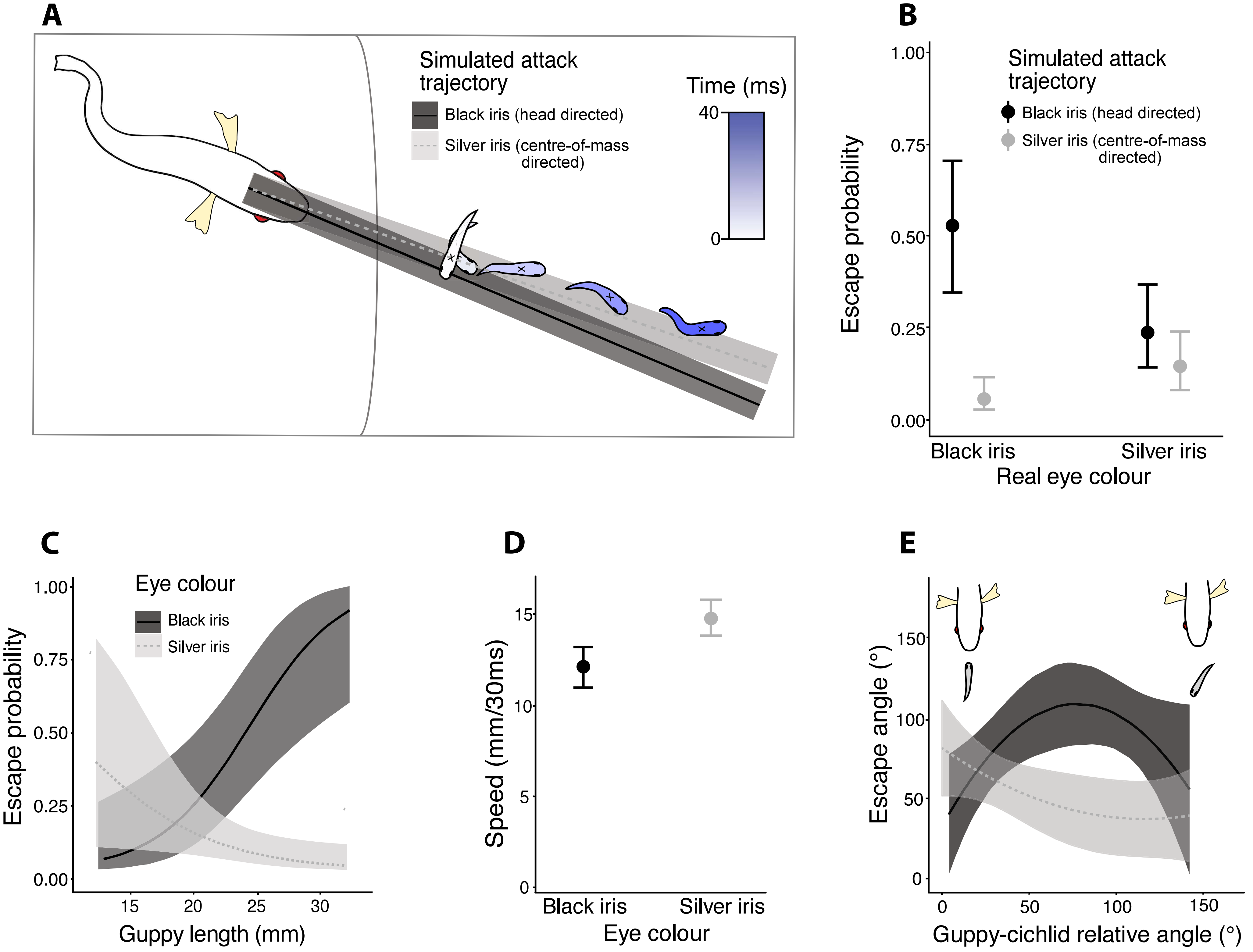
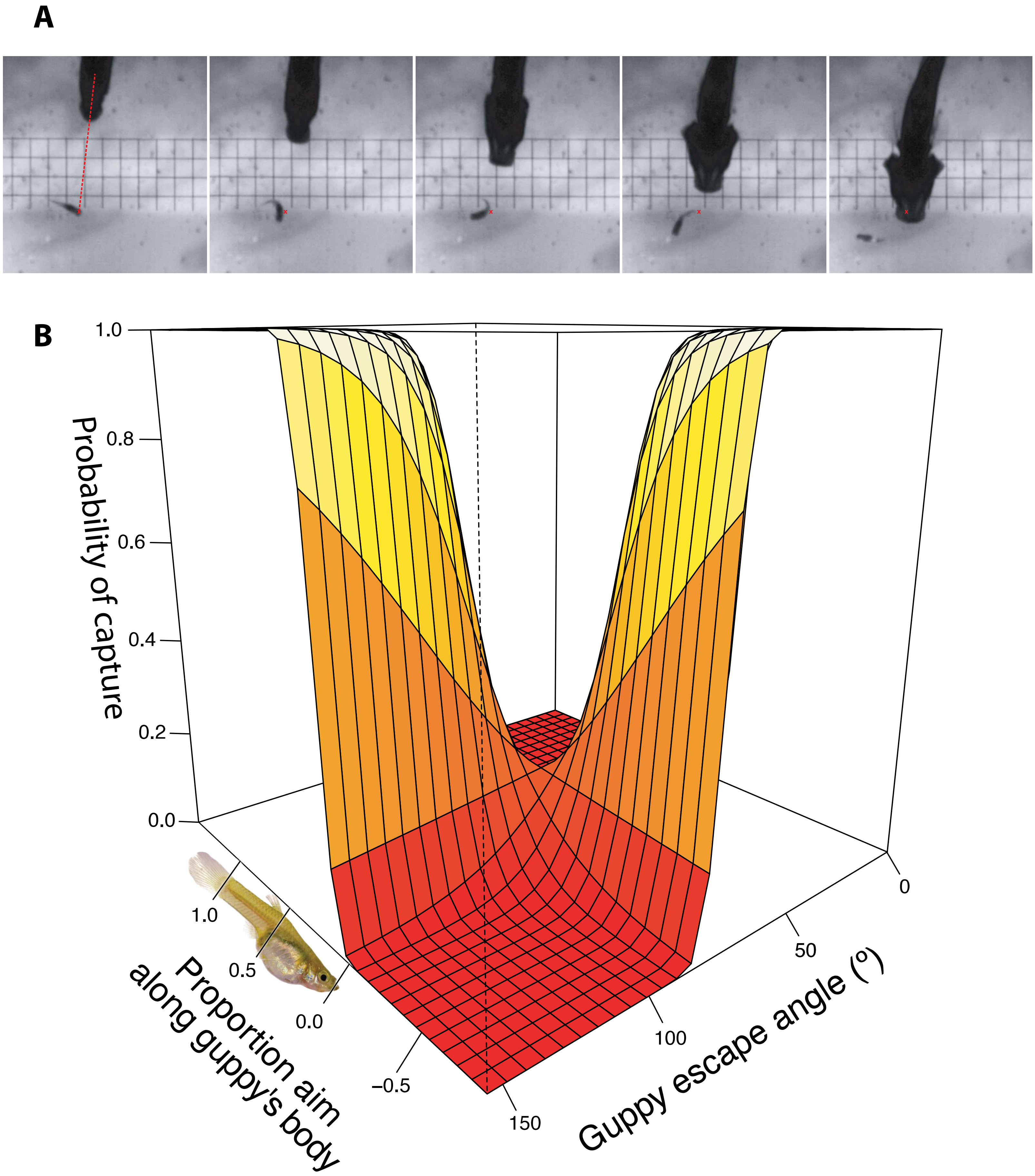

A
Simulated attack trajectory
Black iris (head directed)
Silver iris (centre-of-mass directed)
Time (ms)
40
0
B
Escape probability
1.00
0.75
0.50
0.25
0.00
Simulated attack trajectory
Black iris (head directed)
Silver iris (centre-of-mass directed)
Black iris
Silver iris
Real eye colour
C
Escape probability
1.00
0.75
0.50
0.25
0.00
Eye colour
Black iris
Silver iris
15
20
25
30
Guppy length (mm)
D
Speed (mm/30ms)
15
10
5
0
Black iris
Silver iris
Eye colour
E
Escape angle (°)
150
100
50
0
0
50
100
150
Guppy-cichlid relative angle (°)

Figure 4


A
B
1.0
0.8
0.6
0.4
0.2
0.0
Probability of capture
1.0
0.5
0.0
−0.5
Proportion aim along guppy's body
0
50
100
150
Guppy escape angle (°)

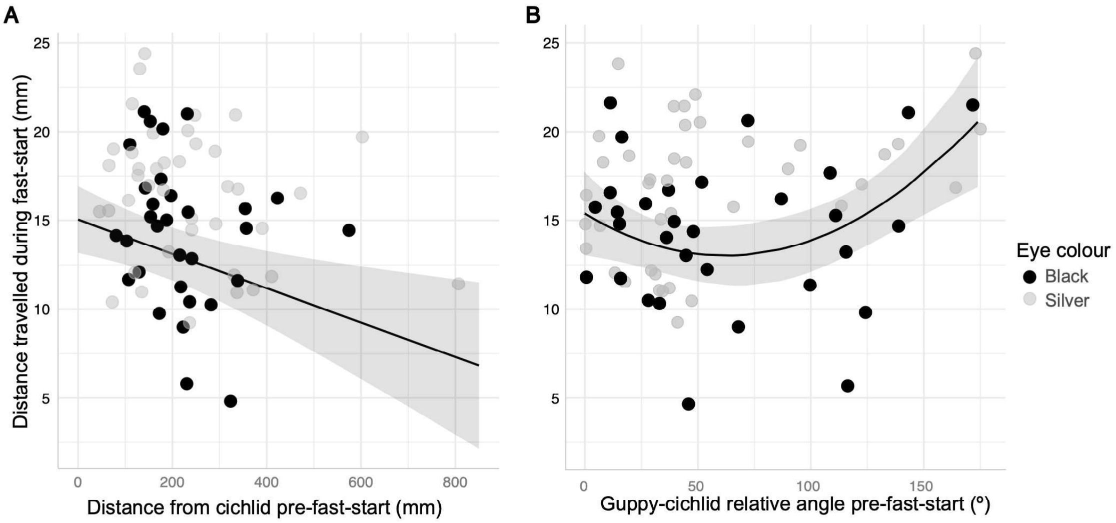


Figure S1. Predictors of guppy fast-start performance in response to pike cichlid attacks during the predation simulation experiment. Related to Figure 3. (**A**) Relationship between guppy speed (mm/30ms) during its fast-start and its distance from the pike cichlid at the time of the attack. (**B**) Relationship between guppy speed and guppy-cichlid starting angle.

Model	Factor	Estimate	SE	χ^2	P
Male	Pred. stim. x Native pred. risk x Body size	-0.93	0.58	2.80	0.094
	Predator stimulus x Native predation risk	0.02	1.04	<0.01	0.988
	Predator stimulus x Body size	-0.07	0.20	0.14	0.707
	Native predation risk x Body size	-0.16	0.28	0.32	0.572
	Body size	0.01	0.14	<0.01	0.951
	Native predation risk	-0.32	0.38	0.71	0.400
	Predator stimulus	0.62	0.38	2.79	0.095
Female	Pred. stim. x Native pred. risk x Body size	-0.11	0.23	0.22	0.639
	Predator stimulus x Body size	-0.03	0.09	0.11	0.735
	Native predation risk x Body size	0.25	0.12	5.38	0.020
	Predator stimulus x Native predation risk	2.09	0.80	7.112	0.008
	Body size	0.03	0.04	0.47	0.492
	Native predation risk	1.17	0.39	7.06	0.008
	Predator stimulus	0.59	0.37	2.52	0.112

Table S1. Summary statistics for predictors of black iris expression in male and female guppies. Related to Figure 1. Estimates refer to log odds ratios. Estimates for the factors ‘predator stimulus’ and ‘native predation risk’ use the control stimulus and low-predation environments, respectively, as reference levels. Statistical information in non-significant terms is included at the point prior to their removal from the model. Information for single terms is included after any significant higher-level interaction has been removed from the model.

Model	**Factor**	**Estimate**	**SE**	χ^2	**P**
Pre-acceleration aim	Eye colour x Guppy-cichlid relative angle	0.00	0.00	0.01	0.942
	Guppy-cichlid relative angle	0.00	0.00	0.29	0.592
	Trial order	0.11	0.11	1.09	0.297
	Eye colour	-0.25	0.10	5.27	0.022
Pre-contact aim	Eye colour x Guppy-cichlid relative angle	<0.01	<0.01	0.09	0.763
	Guppy-cichlid relative angle	<0.01	<0.01	0.25	0.619
	Trial order	0.03	<0.01	0.43	0.510
	Eye colour	-0.16	0.05	9.11	0.003

Table S2. Summary statistics for predictors of aiming point at both pre-acceleration and pre-contact phases of a cichlid's attack on guppy models. Related to Figure 2. Estimates for eye colour use 'silver iris' as the reference level comparison. Statistical information in non-significant terms is included at the point prior to their removal from the model. Information for single terms is included after any significant higher-level interaction has been removed from the model.

Factor	**Estimate**	**SE**	χ^2	**P**
Simulated eye colour x real eye colour x guppy length	-0.18	0.07	6.37	0.012
Real eye colour x guppy length	0.15	0.13	1.36	0.244
Simulated eye colour x real eye colour	-2.38	0.25	99.84	<0.001
Simulated eye colour x guppy length	0.33	0.03	112.15	<0.001
Real eye colour	0.27	0.49	0.29	0.591
Guppy length	-0.01	0.07	0.01	0.990
Simulated eye colour	1.70	0.12	277.16	<0.001

Table S3. Statistical summary of factors that predict the simulated probability of a guppy escaping during the staged cichlid-guppy interactions. Related to Figure 3. Estimates for eye colour use 'silver iris' as the reference level comparison. Statistical information in non-significant terms is included at the point prior to their removal from the model. Results for fixed effects that are involved in higher order significant interactions are included once the interaction has been removed from the model.

Question	**Real:Simulated eye colour combination contrasts**	**Estimate**	**SE**	**Z**	**P**
i. How does eye colour affect survival?	Black:Black - Silver:Black	1.29	0.52	2.50	0.061
	Black:Black - Black:Silver	3.01	0.21	14.21	<0.001
	Black:Black - Silver:Silver	1.91	0.52	3.67	0.001
	Silver:Black - Black:Silver	1.71	0.53	3.23	0.007
	Silver:Black - Silver:Silver	0.62	0.14	4.33	<0.001
	Black:Silver - Silver:Silver	-1.10	0.53	-2.07	0.165
ii. How does body size interact with eye colour to affect survival?	Black:Black - Silver:Black	0.23	0.13	1.74	0.303
	Black:Black - Black:Silver	0.45	0.06	7.80	<0.001
	Black:Black - Silver:Silver	0.50	0.13	3.78	0.001
	Silver:Black – Black:Silver	0.22	0.13	1.63	0.364
	Silver:Black - Silver:Silver	0.27	0.04	6.66	<0.001

Table S4. Estimated marginal means of survival differences based on eye colour and body size based on the simulation experiment. Related to Figure 3. (i) Estimated marginal means comparing differences in guppy escape probabilities across different combinations of real and simulated eye colour contrasts (how does eye colour affect survival?). (ii) Differences in effects of guppy body size between different real and simulated eye-colour combinations (how does body size interact with eye colour to affect survival?). For (i) all comparisons were made when body size was held at its mean of 21.9mm.

Model	**Factor**	**Estimate**	**SE**	**χ^2**	**P**
Distance travelled	Eye colour x guppy cichlid relative angle (linear)	5.15	7.53	2.01	0.365
	Eye colour x guppy cichlid relative angle (quadratic)	-9.05	7.80	-	-
	Guppy length	-0.10	0.12	0.73	0.394
	Distance from cichlid	-0.01	0.01	8.31	0.004
	Guppy cichlid relative angle (linear)	7.71	3.64	12.25	0.002
	Guppy cichlid relative angle (quadratic)	10.89	3.89	-	-
	Guppy eye colour	2.28	0.86	7.10	0.008
Maximum acceleration	Eye colour x guppy cichlid relative angle (linear)	-2.36	3.93	0.96	0.620
	Eye colour x guppy cichlid relative angle (quadratic)	2.83	4.01	-	-
	Guppy length	-0.04	0.07	0.37	0.543
	Eye colour	0.03	0.46	0.01	0.943
	guppy cichlid relative angle (linear)	0.39	1.86	0.83	0.662
	guppy cichlid relative angle (quadratic)	-1.59	1.89	-	-
Escape trajectory	Distance from cichlid	0.01	0.04	0.03	0.864
	Guppy length	0.88	1.58	0.38	0.536
	Eye colour x escape direction x guppy cichlid relative angle (linear)	269.64	197.06	8.06	0.018
	Eye colour x escape direction x guppy cichlid relative angle (quadratic)	-536.06	226.31	-	-
	Eye colour x guppy cichlid relative angle (linear)	-94.38	88.99	5.98	0.050
	Eye colour x guppy cichlid relative angle (quadratic)	196.99	103.28	-	-
	Guppy cichlid relative angle (linear)	-17.43	43.85	0.19	0.911
	Guppy cichlid relative angle (quadratic)	0.42	43.47	-	-
	Eye colour	33.99	13.33	6.63	0.010

Table S5. Statistical summary of factors that predict kinematic performance during a guppy's fast-start when attacked during the predation simulation experiment. Related to Figure 3. Estimates for eye colour use 'silver iris' as the reference level comparison. Statistical information in non-significant terms is included at the point prior to their removal from the model. Results for fixed effects that are involved in higher order significant interactions are included once the interaction has been removed from the model.

Factor	**Estimate**	**SE**	χ^2	**P**
Escape trajectory x Aiming point x relative angle	-0.01	0.01	2.89	0.089
Aiming point x relative angle	-0.07	0.07	1.24	0.265
Escape trajectory x relative angle	<0.01	<0.01	1.32	0.251
Relative angle	-0.01	<0.01	0.80	0.372
Escape trajectory x Aiming point	0.56	0.21	22.69	<0.001
Escape trajectory	0.01	0.01	0.28	0.596
Aiming point	-0.47	0.91	0.27	0.604

Table S6. Statistical summary of factors that predict probability of a guppy being eaten during the live predation interactions. Related to Figure 4. Non-significant values are included at the point prior to their removal from the model. Results for fixed effects that are involved in higher order significant interactions are included once the interaction has been removed from the model.