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February 1, 2023

How to generate and test hypotheses about colour: insights from half a century of guppy research

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Complex colouration facilitates evolutionary investigations in nature because the interaction between genotype, phenotype and environment is relatively accessible. In a landmark set of studies, Endler addressed this complexity by demonstrating that the evolution male Trinidadian guppy coloration is shaped by the local balance between selection for mate attractiveness versus crypsis. This became a textbook paradigm for how antagonistic selective pressures shape evolutionary trajectory. However, the paradigm's generality was recently questioned by two studies seeking to use Trinidadian guppy populations to test the concept of parallel evolution. Both applied new and more sophisticated colour pattern analysis but these studies neither adequately address the question of evolutionary parallelism nor actually challenge the paradigm. As a guide to future work we review five main underappreciated factors contributing to colour pattern evolution: (1) inter-population variation in female preference, (2) differences in how predators versus conspecifics view males, (3) biased assessment of pigmentary versus structural colouration, (4) the importance of accounting for multi-species predator communities, and (5) the importance of considering the multivariate genetic architecture and multivariate context of selection. We elaborate upon these points and emphasize the depth of consideration necessary for testing evolutionary hypotheses using complex multi-trait phenotypes such as guppy colour patterns.

Key Words: Colour Patterns, Colour Pattern Evolution, Colour Pattern Analysis, Colour Pattern Perception, Avoiding Analytic Errors

1. Introduction

Complex colouration can facilitate evolutionary investigations in nature because the interaction between genotype, phenotype and environment is relatively accessible. Humans and other animals differ in their visual abilities and perception; consequently biologists invariably experience things differently than the species they study [1, 2]. It is therefore challenging to represent visual phenomena in the terms that matter to natural viewers [2, 3] and how to formulate and test appropriate hypotheses [4]. A clearly defined research question is of paramount importance, yet its rationale must also explicitly account for which viewers are involved, their visual systems and visual perception, their visual signal properties, the viewing environmental conditions, and any behaviours that may characterize how such phenotypes function [2, 3, 5, 6]. For meaningful colour-based research we need to understand how animals see and interact with the world.

Here we provide a perspective on developing and testing hypotheses about non-human visual perception and function. First we summarise some investigative pitfalls, then describe factors affecting colouration, and use it as way to encourage broader and more complete colour pattern investigations. We explore these issues by tracing efforts to understand the evolution of ornamental colouration in wild guppies, *Poecilia reticulata* Peters. Guppies offer a rich medium for discussion because a great deal is known about their visual system, behavioural ecology, and evolution [7-14]. The literature devoted to the study of guppy colouration also echoes the development of the tools for quantifying colour patterns [2, 3, 5, 11], including efforts to apply the very latest colour analytics [15, 16]. This extensive history of sensory-oriented guppy research presents a timely narrative for how to maintain intellectual rigor in the face of potentially transformative advances in analytical capacity.

2. The guppy colour evolution paradigm

Guppies are small freshwater fish native to north-east South America and adjacent islands, where they occupy shallow, clear-water rainforest streams with visually diverse backgrounds comprising multi-coloured gravel, rocks and leaf litter [2]. This species has proven popular for studying animal colouration because males possess extensively polymorphic colour patterns [2, 13, 17, 18]. These patterns consist of discrete pigment-based orange, yellow and black spots, less discrete but temporally dynamic melanic markings [19], called “fuzzy black” [2], and structural colours. The latter class of markings appear to the human viewer as bright flashes of violet, purple, blue, teal, green, and silver, which often grade into each

other across the flank and/or with changing angles of view (Figure 1). Guppies and members of their potential viewing audience, such as predatory *Macrobrachium* prawns, perceive a greater range of hues because they can perceive the strong UV reflectance of some colour patches [20-24]. Males bear these complex colour patterns as a consequence of female mating preferences [25]. Guppies also naturally exist with a variety of visually-orienting predators, which would otherwise be expected to penalize such conspicuousness [2, 26]. Consequently, male guppy colouration is the product of a trade-off between sexual selection, favouring mate attractiveness, versus natural selection, favouring crypsis [2, 27, 28].

Endler's studies of male guppy colouration in the late 1970s [2, 27, 28] produced a textbook paradigm of experimental evolution in the wild. Briefly, evolution was manipulated in two ways. First, Endler transplanted guppies from a downstream predator-rich locality on the Aripo River to an upstream site that excluded virtually all predators, including the most dangerous visually-orienting species *Crenicichla frenata* (formerly *C. alta*). The experimental population therefore experienced a reduction in predation risk, which gave rise to a situation where female preference for male ornamentation prevailed. Endler replicated this experiment in replicate artificial streams in which he manipulated predation risk in both directions by having streams with no predators, reduced predation (only the killifish *Rivulus hartii* present) or high predation (*C. frenata* present) (Fig. 1 in [27]). Both experiments revealed that added risk of predation caused reduced male ornamentation while reduced risk caused increased ornamentation. Changes in response to reduced predation risk were most evident via increases in the number and/or size of structurally-coloured blue, silver and bronze spots, as well as increases in the size of pigmentary black and orange spots. Structural colours are highly relevant to predation risk because they generate conspicuous flashes of colour that are visible from a distance, even under low light of the rainforest floor [2, 28]. Larger spots are also more risky because they are more likely to exceed the chromatic and achromatic visual acuity thresholds of predators and thereby increase the distance at which guppies may be first detected by predators. These results were particularly compelling because they corresponded closely with how guppy male phenotypes vary across locations throughout Trinidad and Venezuela that presented these same differences in predation risk [2].

Investigations of guppy colouration in both natural and experimental populations have since supported this paradigm (e.g., [14, 23, 24]). However, its generality has been questioned by two recent studies [15, 16] that use guppies in two new methods of colour pattern analysis. A unifying motive for both studies was to generate a more holistic quantification of the male colour pattern as opposed to atomizing it into counts and sizes of discrete colour patches, as pioneered by Endler [2, 27]. Yong *et al.* [15] applied one component of a framework termed “Quantitative Colour Pattern Aalysis” (QCPA) [29]. This application of QCPA focused upon the visual saliency of boundaries (edges between adjacent colour

patches) [29]. Valvo *et al.* [16] used a tool termed “colourmesh”, which characterizes colour at homologous locations across the organism. Both studies present serious attempts at objectively capturing the spatio-chromatic detail of complex colour patterns, although Valvo *et al.* [16] does not account for sensory aspects of coloration. Both address in different ways whether or not guppy colour phenotypes evolve consistently under equivalent predation intensities. Each study defined evolutionary trajectories as the vectors joining centroids for high predation populations to those of low predation populations in the same river. They then tested whether or not the vectors representing differences between high and low predation communities from different rivers are improbably similar to each other in length and direction. Both authors conclude that they are not and they therefore do not reveal convergent evolution of male colouration as guppies adapt to high versus low predation environments. This has been interpreted by them as evidence for a lack of “parallel evolution” [15, 16], meaning that adaptation of the guppy colour phenotype may be neither replicable nor predictable – thereby contradicting the very basis of Endler’s paradigm [2, 27, 28].

Here we show how the results of these studies do not in fact contradict the established paradigm and illustrate the difficulty of colour pattern investigations. This follows from practical issues regarding guppy sensory, behavioural and community ecology, and theoretical issues relating to evolutionary genetics, multivariate trait evolution [30, 31] and the nature of parallel evolution itself [46]. After a brief summary, we will discuss five main points which collectively underscore the depth of consideration necessary for testing evolutionary hypotheses using complex multi-trait phenotypes such as guppy colour patterns:

- (1) *Variation in selection*: Given that colouration is conceptualized as a balance between opposing multivariate vectors of sexual and natural selection, population-level contrasts must consider the potential for variation in all sources and targets of selection. The phenotypic target of guppy female choice varies within and among rivers and within populations [32, 33], as it does in other animals [34]. Differences or changes in predation across populations may therefore see sexual selection favour any number of possible routes to increased visual conspicuousness [32, 35]. Moreover, the complex and spatially variable visual backgrounds in these streams means that there are a large number of ways of being equally visible, even within a population [2,3]. This could easily promote varied evolutionary trajectories even under constant predation risk.
- (2) *Visual acuity and motion*: The distance from which colour phenotypes are seen and the visual acuity of the observer will determine which features are most salient. Predators detect male guppies from distances that generally preclude their ability to resolve the intricacy of colour patterns *per se*, whereas females appraise courting males at close range [11, 36]. This difference in viewing distance is key to considering how colouration may be influenced by natural versus sexual

selection [2]. Motion blur further modifies the appearance of a colour pattern, sometimes profoundly, depending upon motion direction.

- (3) *Structural colour visibility*: Structural colours are often highly visible over long distances and therefore particularly relevant to predation risk [2]. Since they flash during courtship, they are visible under lower light levels than pigment and melanin-based colours. Dedicated measurement protocols are required to properly estimate these visual features.
- (4) *Viewer sensory properties*: Sensory-based analyses will only yield accurate conclusions and predictions if such analyses include appropriate visual parameters, including effects of perception as well as eye functions. For heterospecific viewers such as predators this requires that relevant species are correctly identified and that their vision be characterized [11]. Further, if multiple species of predators are present, the relative risk posed by each species should ideally be weighted to derive a composite risk estimate of how prey colour patterns are likely to experience natural selection (as in [2, 11]).
- (5) *Multivariate selection and genetic response*. It is unrealistic to assume that selection should always engender similar change across all colour pattern component traits. This is true at three levels: individual trait function, phenotypic selection and genetic response. Each trait should have its own array of natural and sexual selection effects as well as potential evolutionary influences modulated by multivariate genetic architecture. Consequently, covariances across the phenotypic selection variance-covariance matrix should not be assumed to be high and positive, but instead to range anywhere between -1 and 1. Similarly we must not assume that the genetic covariances are all positive. Consequently, the estimated balance between sexual selection and natural selection for crypsis will depend upon which traits are included in the estimation of colour phenotype and how they are selectively and genetically correlated.

2.1. Variation in selection

Endler's (1980) classic guppy evolution experiments have held great appeal because they illustrate a fundamental theoretical tenet: levels of sexual ornament expression should be balanced against naturally-selected viability costs [37]. For guppies, this suggests that selection for net lifetime fitness should favour increasingly "colourful" male phenotypes up until the point where the benefits of sexual attractiveness become offset by the predation risk. By experimentally reducing predation intensity in the wild, Endler [27] shifted the local balance between sexual and natural selection, thereby freeing male colour ornamentation to evolve more conspicuousness. Sexual selection should drive evolutionary change in because females judge mate attractiveness relative to their options in their population. The fact that sexual selection favours relative phenotypic targets, rather than absolute ones, is important because this provides

the potential for idiosyncratic or unique evolutionary trajectories, thereby contributing to population divergence.

The guppy evolution paradigm has since been generalized to predict that populations subject to relatively low predation risk should be more colourful than those under greater risk [22, 38, 39]. This proposition is reasonable, but only in the broadest sense. It does not mean that all low predation populations should necessarily evolve increased colourfulness in the same way. There is enormous complexity in the potential colour phenotypes/traits that may prove attractive to females. Even within a given population, the visually diverse nature of guppy habitats presents literally hundreds of different ways that a male colour pattern could achieve the same degree of conspicuousness [2]. This diversity is compounded by variation among individual females in what they find most attractive [33], including their potential penchant for novelty [40-43]. It is also well known that female preference varies among guppy populations. [32, 35, 39, 44]. Endler & Houde [32, 35] demonstrated substantial variation in the target(s) of female preference across 11 populations from nine different Trinidadian rivers. They also found that the manner in which colour phenotypes were enhanced in low predation environments differed among rivers and did so in ways commensurate with local female preferences. The guppy system is very permissive of variation in appearance, and hence permissive of population divergence shaped by the different combinations of visual backgrounds, lighting conditions [45, 46] and female preferences that exist within and among localities. This is in fact one of the explanations for their extreme polymorphism [2].

The variation in how guppies adapt to low predation environments has also been demonstrated via transplantation experiments conducted in different rivers. The evolution of male phenotypes in response to transplantation from predator-rich to previously guppy free, predator-poor environments differed among rivers in ways that align with differences among rivers in female preference [24]. In Endler's original experiment in the Aripo river, males from transplanted populations evolved larger pigmentary and structural coloured markings (Fig. 3 in [27]). By contrast, when the same experiment was done in the El Cedro River, males evolved smaller pigmentary spots coupled with greater areal coverage and reflectance intensity of structural colour (see Figs. 2 – 3 in [24], also [14]). Such outcomes imply that differences among rivers in how sexual selection is manifested will cause divergent vectors of colour exaggeration. Divergence among populations experiencing similar selective conditions is expected and accommodated by modern views of parallel evolution [47] which expects a range of patterns between strong (little within regime variation) and no parallel evolution. This continuum is expected among traits, as well as populations, so we should not expect different traits to show the same pattern.

2.2. Visual acuity and motion

Efforts to infer how particular viewers may select colour patterns have considered spectral sensitivity more often than visual acuity [48,52]. Neglecting spatial acuity will only yield reliable conclusions when colour patterns are viewed from close range. This is because spatial resolving power (acuity) – a product of both visual acuity and viewing distance – determines the extent to which a viewer can appreciate the full detail of a colour pattern and therefore its degree of visual contrast [2,36,52]. Acuity has general relevance for appraising how colour patterns could appear to conspecifics compared to predators.

Predators detect prey from greater distances than conspecifics observe each other during courtship. Endler [11] estimated predator attack distances from direct observations in natural streams. Predators viewed guppies at distances ranging from five (*Rivulus*, *Macrobrachium*) to twenty times (*Crenicichla*) the distance that female guppies typically view courting males (ca. 20 mm). This difference in viewing range means that fine-scale features of male colour pattern, such as small spots, lines and colour patch boundaries, are far more likely to have salience to courted females than to predators [2, 36]. Likewise, the boundaries between otherwise highly contrasting features of the colour pattern are likely to become increasingly blurred with increasing viewing distance and/or decreasing visual acuity, see Figure 2A,B. It is thus critical to account for known predator attack ranges (i.e. retina to guppy distances [11]) and visual acuity [2, 36,48] to determine how colour patterns will be perceived, and selected [2,52].

Motion blur occurs when the response time of photoreceptors is too slow to respond to fluctuating inputs as the visual field changes during motion; visual acuity decreases along the axis of motion [49]. Figure 2 shows how motion blur can profoundly affect a guppy's appearance. Rotation around the body long axis during the sigmoid courtship display [18] results in vertical motion blur leaving fine detail (Figure 2C). Movement along the body long axis during foraging or predator escape leads to an entirely different pattern (Figure 2D). Spots elongated in the direction of movement are less subject to motion blur than when motion is perpendicular to their elongation. Long patches may also make it difficult for a predator to track the guppy, as in striped snakes [2]. Bright colours in bars can have greatly reduced colour contrast when motion blended, as in coral snakes and their mimics [50]. Consequently, the same colour pattern can appear very different to guppies and predators, depending upon how and when the guppy moves and the visual abilities of each predator. More work on the effects of motion blur relative to colour pattern geometry would be valuable.

Predator spatial acuity is highly relevant to approaches like Yong et al.'s (14), who focused on colour patch boundaries to analyse the visibility/detectability of colour patterns to different viewers. Recent work in guppies [51] reveals that boundary strength (a measure of contrast between adjacent colour

patches) explained between 34 and 70 % of variation in female guppy mating decisions. This makes intuitive sense, given the close range from which females observe males. In contrast, pattern edges contribute only weakly to stimulus detection by predators; for example, boundary contrast explained at best only 6 – 8 % of predator attack success in the triggerfish (*Rhinecanthus aculeatus*) [52]. Troscianko *et al.* [53] also found low predictive power of boundary-related traits for human search for items against a background. These analyses accounted for natural attack range. These results were statistically significant, although statistical and biological significance are not the same. It thus appears that relying heavily on colour patch adjacency and boundary strength by itself does not best represent how predators detect prey, even if it works for mate choice. This suggests why Yong's *et al.* analysis of guppy colour pattern edges and predation levels yielded minimal effects. It may well be that patch characteristics are important for predation and mate choice whereas patch boundaries are only important for courtship, but this needs further investigation.

Detectability is also influenced by how colour patches deviate from the spatial grain of the viewing background [2, 27], and this may result in different components attracting attention at different distances. Moreover, tannins and other pigments in the water and the colour of the spacelight will cause some colours to blend with the background whereas others will remain conspicuous [11]. Colour detection becomes more difficult as patch size decreases and this decline is faster as light intensity decreases. Endler [27] formally tested the importance of backgrounds in shaping colour pattern evolution by lining his artificial streams with either fine or coarse multicoloured gravel and hence manipulating the background against which predators perceived guppies. When predators were present, larger gravel was associated with the evolution of significantly larger spots in male guppies whereas smaller gravel favoured smaller spots. The reverse was true when predators were absent (Fig. 3 in [27]). Here there was significant divergence within predation regimes directly related to visibility on local backgrounds.

2.3. Structural colour visibility

Endler [2] observed that predators are most likely to detect male guppies via their highly reflective structural colours, which generate intense “flashes” of colour visible over long distances (as noted in other animal groups; e.g., [54]). Guppies produce such colours via arrangements of micro-platelets situated in the iridophore cell layer [10] that scatter light of select wavebands across a restricted angular range and are highly efficient reflectors. The visibility of guppy structural colours depends on the angles that they are viewed and illuminated from. In the shallow water of guppy habitats illumination comes from above in a relatively narrow angle called Snell's window [55] which can enhance the effects of structural colours since reflectivity is strongly dependent upon illumination and viewing angles [54,55,59].

Structural colours also vary subtly in colour (hue) depending upon the precise orientation from which they are viewed and illuminated, such that the same region of flank may appear greenish-blue, teal, or bluish-green when seen from different angles. Many structural colours reflect in the ultraviolet, and this often underlies the pigmentary red/orange/yellow spots that occur across the guppy flank ([23, 24]; Fig.1 in [15]). The structural UV may serve to visually amplify [56] these longer-wave pigmentary colours [10, 57], or vice-versa [58]. More generally, the coincidence of orange pigment and structural UV means that such colour patches are visually dynamic; their apparent hue and/or luminosity may shift markedly with even subtle changes in viewing angle or when the guppy moves between shade (no UV) and direct sunlight (UV rich). This variation is enhanced by the way male displays involve rotation of the body axis, which induces fluctuations in both colour and intensity.

The features that make structural colours visually dynamic also make them challenging to quantify. Their only being visible from restricted angles, for example, means they cannot be effectively measured using a fixed photography or spectrometry configuration. This is because different individuals may vary in the orientation at which their structural colouration is visible. Simply photographing all guppies at a single 'standard' orientation is likely to underestimate structural reflectance across fish, due to varying degrees depending on how far the setup displaces each individual from its optimal camera viewing orientation. Irrespective of the recording design, illumination should come dorsally, as from Snell's window, and the camera or sensor should be positioned to emulate the orientation from which females and/or predators naturally view males. Predator and female viewing angles can be different and should be accommodated.

Given that structural colours arise from selective reflectance (as opposed to selective absorption), their visual effect is often accentuated by the co-occurrence of pigments (and/or vice-versa) [57, 58]. Underlying layers of melanin pigment, for example, absorb broadband light which would otherwise reflect from or transmit through the signalling surface, thereby acting in the manner of how a blackboard gives visibility to chalk. Guppy structural colours are unusual because they exist across largely unpigmented regions of the male's flank. The suffusion of iridescent violets, blues and greens across the posterior flank and caudal peduncle is often exclusive rather than inclusive of the black spots and dynamic fuzzy black markings that occur in this region (Figure 1). This makes for potentially high within-pattern contrast, yet the degree of such contrast depends on both the angle at which the flank is viewed as well as the immediate viewing background. Structural colours will be most obvious when minimal light is propagated from behind; that is, when an individual is viewed against a dark background, (Figure 1) such as the water column [46]. Such colours will engender the most startling visual effects when a male is lit by a narrow shaft of sunlight, which might often occur under dappled forest light. This is especially true early or late in the day, when low solar angles predominate. This is also when male guppies are more

likely to be courting females [7]. Such scenarios therefore enable courting males to present their structurally-coloured phenotype to greatest effect, but are also when they will be most vulnerable to long-range detection by predators (*sensu* [2, 27, 28]); although there are ways around this [46]. It is critical to emulate this visual geometry to faithfully account for the biological consequence of structural colouration [59].

Endler (1978) addressed these complexities using multiple assessment methods. He first observed unanaesthetised fish in natural daylight, which means that he saw them from different angles and against different backgrounds as they moved and was able to record the presence of all structural colours. He then photographed all fish to measure spot size. Virtually all subsequent research on guppy colouration (e.g., [15, 16, 22, 33, 38, 42]) is based entirely on data from photographs taken against white backgrounds, which certainly underestimates the contribution of structural colour. A relatively simple way to enhance the visualization of structural colour is to photograph the fish on both white and black backgrounds while ensuring that the geometry of lighting and camera/sensor replicates what female guppies see in the wild. White provides a good background for perceiving pigment-based markings while black is more effective in highlighting structural colour [14] (Figure 1). Images captured against a white background will systematically underestimate the occurrence of structural colour and, by extension, its potential visual interaction with pigmentary markings.

The colouration of male guppies from the Guanapo River and its tributaries, including the El Cedro River [24], illustrate the potential danger of not properly accounting for structural colouration. Males from these and other Trinidadian locations display pronounced structural violet, blue and green (+ ultraviolet) markings. As noted earlier, adaptation under experimentally relaxed predation risk in the El Cedro River and four other Guanapo tributaries resulted in the enhancement of these markings at the expense of pigmentary colours [14, 24], which is fundamentally different from the evolutionary response observed by Endler in his Aripo experiment [27]. In rivers like the Guanapo and El Cedro, where adaptation to low predation and low light levels primarily involves the enhancement of structural colour, failing to properly account for such colouration has led past authors [15, 16, 22] to conclude that adaptation to low predation generates less colourful ornamentation.

2.4. Viewer sensory properties

Correct inferences about the selective effects of predation demand an accurate representation of which predators are present/absent across focal study sites. This is even more important when sensory-based modelling is employed to estimate prey visual conspicuousness. This is because different predator species vary in visual capacity [11] – including spectral sensitivity and spatial acuity – but also because of

differences in behaviour and ecology that determine the visual context under which they operate. For example, locations with only *Rivulus* and/or prawns tend to have closed or nearly closed canopies and a different set of light conditions to the more open areas where *Crenicichla* are found, although canopy closure varies even within predator locations [14]. Diurnal predators will obviously differ from nocturnal predators in how they select prey colouration. Likewise, characteristic attack range will determine the potential visibility of prey colour patterns and dictate the level at which selection may operate upon them. Despite these qualifications, there is a significant tendency for loose assumption of the natural history context of prey colour evolution, including the substitution of proxies for predatory species when information is otherwise lacking. We detail below how this tendency is evident in the recent tests of parallel evolution in guppies [15, 16].

Endler [2] conducted timed visual censuses of the predator community at all sampling sites [7] so that each site could be associated with a list of the species of diurnal predators and their relative abundance. He also quantified the attack rate of the different predatory species, and then developed an index to estimate the severity of each guppy predator species. He found a gradient of predation as one progressed from downstream to upstream. The total number of fish species and the number and abundance of species that prey on guppies is greatest downstream and progressively declines upstream. For example, *C. frenata* (the most dangerous guppy predator) was also one of the species most likely to have its upstream distribution stopped by natural barriers like waterfalls. This gradient in predator communities was matched by a gradient of male colouration, as assessed by variables such as spot number and spot size across the different colour categories (see Table 3 & Figs. 11–13 in [2]). In contrast, [15] and [16] report no assessments of predation and instead simply classify the communities dichotomously as either low or high predation risk. This is problematic because the composition of the community will affect the expected magnitude of the selective differences between their high and low predation localities. The dichotomous treatment of predator communities was first associated with research on life history evolution [60], where it was based on a deliberate restriction of study sites to those that either had the full complement of potential predators versus those where guppies co-occurred with only *R. hartii*, which feeds primarily on invertebrates but will also prey opportunistically on vertebrate prey, including guppies [61–63]. Subsequent work has revealed a gradient of life history traits, like fecundity, that matches the gradient in predator communities [64].

Some Trinidadian river communities have changed dramatically in the 45 years since Endler’s work. In fact, changes were even noted in the late 1980s [65]. The Guanapo, for example, now has a large, active quarry that deposits abundant sediments in the river. Ehlman *et al.* [66] replicated prior census techniques [62] to show that *Crenicichla* abundance has declined dramatically over the past 25 years in the Guanapo,

which was included in the studies of Yong *et al.* [15] and Valvo *et al.* [16]. Given the known rate at which male colour patterns can evolve [27], it is conceivable that the differences among guppies from low and high predation communities now may be different from what they were during Endler's studies.

Yong *et al.* [15] and Valvo *et al.* [16] include sites on the north slope of the northern Trinidad mountain range, where there is a different community of predators than exist throughout the drainages on the south slope. Rivers on the south slope were once tributaries of South American Rivers and are dominated by cichlids and characins. Rivers on the north slope are dominated by fish with a marine origin; cichlids and characins are almost always absent. The primary predators are Gobies (*Gobiomorus*, *Dormitator*), mountain mullet (*Agonostomus*) and the diurnal freshwater prawn *Macrobrachium crenulatum*, which has very different colour vision from fish [2]. There are no published assessments of the fish communities of the north slope rivers comparable to those of the south slope, so the distribution and abundance of the key predators is poorly characterized (but see [2] and [65]). Many reports of life histories and male colouration that are inconsistent with the expected differences between high and low predation communities (e.g., [22, 67] are based on work done on the north slope rivers without the benefit of any attempt to assess abundance, distribution or risk of predators. It is essential in hypothesis testing to have an accurate assessment of causal factors.

2.5. Multivariate selection and genetic response

In studies of complex multi-trait phenotypes like guppy colour patterns it is unreasonable to assume that selection will influence all components of male colour patterns in similar ways across populations. There are three main reasons: different traits are subject to different selective factors, the form of the selection variance-covariance matrix among the traits, and the form of the genetic variance-covariance matrix.

Individual colour pattern components are targeted differently by different selective factors. This is strongly suggested by patch boundaries being important in guppy mate choice [51] but apparently not in predation risk [15]. One possible explanation for this is that guppy courtship takes place close enough for very fine detail to be seen [36] but all predators attack from a much longer distance and have lower acuity and more restricted colour vision [11], as in Figure 2B. With greater distance and lower acuity, either boundaries would blur or the differences on either side of the blurred boundary would be greatly reduced, rendering boundaries less salient to predators. Predators would still perceive larger patches and colour contrast, even if reduced compared to what female guppies see.

Each male colour trait will be selected in different ways depending upon the viewer and visual conditions under which the colour pattern is seen. Hence, differences in female preference among populations will

engender a different selective profile. Likewise, predators that possess different visual systems will induce natural selection for crypsis in different ways causing variation in selection as a function of geographic variation in predator communities. The consequence such variation can be seen in guppies from populations that coexist with predatory prawns, who are unique among guppy predators in their ability to see in the ultraviolet but not in the red-orange range of the spectrum [2]. Male guppies in these populations have smaller and/or less reflective ultraviolet markings but more extensive orange [23, 38], as expected if such traits were selected through the prawn visual system. Additional local factors such as canopy openness, ambient light colour [46], water clarity, water colour, and background type may bias colour perception in site-specific ways (e.g., [14]). Each trait is therefore likely to have a different array of natural and sexual selection affects and different "targets" and directions of selection. Some traits will evolve due to sexual selection and not predation, others will evolve due to predation and not sexual selection, and others will evolve in response to both but to varying degrees depending upon the general biology, physics, and the habitat variation among and within streams. Moreover, the same traits may respond differently to different viewers and motion contexts (Figure 2).

There is an unfortunate implicit assumption in the literature that selection is the same for all components of a colour pattern. This ignores the multivariate effects of selection, particularly the effects of correlational selection, where selection on one trait may be positively or negatively correlated with selection on another trait [31]. Correlational selection is summarized by the selection variance-covariance matrix (γ) which also includes the effects of nonlinear (disruptive or stabilizing) selection [68]. These effects are ignored in most studies but are potentially important because they can affect evolutionary trajectories [31] in unexpected ways, especially when γ contains both positive and negative covariances and varies among populations. The action of γ can also cause the genetic architecture to evolve in particular directions, especially if there are negative selective covariances [31]. Further evolutionary constraints can be caused by stabilizing or disruptive selection as estimated by γ [31, 68].

Even if there were no selective covariances or nonlinear selection [68], multivariate evolution can be influenced by genetic architecture (summarized by the genetic variance-covariance matrix, G). If some traits are negatively genetically correlated, then selection on one trait can cause opposite changes in another trait, and, if positively correlated, selection on one could yield changes in another even if not selected. Moreover, the form of γ can affect the evolution of G , further affecting the course of evolution. Variation will be generated by among-population differences in either or both γ or G , neither of which have been investigated in guppies. Generally, phenotypic selective and genetic associations among traits may either facilitate or impede adaptation. Genetic architecture should have a major effect on how colour

patterns evolve and diverge, meaning that a multivariate trait approach should be central in efforts to understand parallel evolution [69].

Consideration of guppy colour patterns as multivariate traits suggests that study conclusions will be highly dependent upon which traits are included in the estimation of colour phenotype (an issue discussed earlier regarding structural colouration). It also poses major problems for studies such as [15] and [16] that seek to dichotomize sites as simply “high” and “low” predation risk. Predation risk is a quantitative phenomenon that will induce different quantitative intensities of natural selection upon each of the traits that constitute the overall colour pattern. Moreover, parallel evolution itself is manifest in a range of intensities owing to the common appearance of within-environment divergence [47], so we have to be very careful in interpreting statistical tests for parallelism. We cannot regard parallel evolution as absent or present, especially when there is diversity of evolutionary trajectories within a given environment [46], as in guppies [2,26,31].

3. How do different methods compare?

Yong *et al.* [15] and Valvo *et al.* [16] both conclude that their results differ fundamentally from Endler’s, yet they reached this conclusion via different methods that accounted for fewer and different traits than Endler did [27]. It is therefore fair to ask how their and Endler’s results compare if performed on the same data. Here we report an application of Endler’s and Valvo *et al.*’s methodologies to a common series of photographs. We used Endler’s original photographs of males from high/low predation environments on the Aripo and Arima Rivers [2]. The high predation environments were ones that had the full spectrum of diurnal predators (*Crenicichla alta*, *Aequidens pulcher*, *Hoplias malabaricus*, *Astyanax bimaculatus*, *Hemibrycon dentatum*). The low predation sites had only the killifish *Rivulus hartii*. Because we were working with photographs of fish on a white background, we only enumerated the number and size of orange and black spots (see section 2.3). Our results for the Endler analysis parallel his original results, which are that the representative males from low predation environments have larger orange and black spots and more black spots than their counterparts from high predation environments downstream ([2], Figure 3a–b). Neither we nor Endler found more orange spots in low predation environments. Overall, the differences support Endler’s original conclusion that males from low predation environments are more highly ornamented than those from high predation environments in the same stream. Note that this test is only a partial assessment of male colouration because we were not able to characterize structural colour from these photographs, and neither study assessed patch boundary strength.

We applied Valvo *et al.*’s methods [16] to this same set of photographs. We found, as did Valvo *et al.*, that one discriminant function discriminates among rivers, but we did not find a single function that

discriminated between high and low predation (Figure 3c–d). In addition, we performed their bootstrap analysis for parallelism using the R-code they provided and found that the distribution of vector correlations strongly overlaps zero. That is, changes in the two rivers are not parallel.

How do we resolve this apparent contradiction in results and conclusions? First, Endler and Valvo et al. (and Yong et al.) measured very different things. Endler measured spot size and number grouped by colour class. Valvo et al. measured the colour of specific pixels across the body. Although the latter will be influenced by the former, it includes positional information as well. Second, the two methods have different expectations and interpretations. Endler tested for a change in spot size and spot number *on average* between different predation regimes while Valvo et al. (and Yong et al.) tested for whether or not the magnitude of the difference between high and low predation was the same in each pair of streams. They included such paired comparisons for four streams and hence made six paired comparisons. Endler’s interpretation of increased ornamentation in low-predation vs high-predation regimes is agnostic on the specific changes (i.e., which colours) precisely because this was already known to vary among streams (as implied in Fig. 7 of [2] and made explicit in [32]). Given this, the remarkable finding is not that Valvo et al. failed to find parallel changes in 4 of 6 pair-wise comparisons, but that they did find significant correlation in 2 cases (the El Cedro-Guanapo and Aripo-Turure). Again, this is at least partially due to measuring very different traits with very different effects on guppy versus predator vision and perception. It is not at all surprising that predators do not select for increased boundary contrast, provided that they can even see the boundaries given acuity effects of visual acuity, attack distance, and water transmission. In contrast, guppies can see all aspects of colour patterns in fine detail [36, 46]. Finally, we note that parallel evolution is not an all or nothing phenomenon and comes in varying degrees of intensity within and among both species and traits [46].

4. General factors affecting the function and evolution of colour patterns

We have identified pitfalls in guppy colouration research, but this gives only a partial picture of all of the factors which affect the function and evolution of colour patterns. Consequently, we provide a list of multiple factors which need to be considered seriously when studying colour patterns of any species (Table 1). It is organised in the order in which light travels from the sun, environment and visual target through the eye to the brain and decision making. Further discussions are found in [2–4, 6, 29, 55]. This is not an exhaustive list but provides a starting framework for serious study of colour patterns. There is very high variation in the amount of investigation of each item in this list, and we hope it will encourage research in neglected aspects of colour patterns, such as the effect of motion blur and differing perception of the same colouration by different species viewing the same object.

5. Conclusions

The first studies of ornament evolution in guppies [2, 27, 28] set high standards in terms of the number of localities sampled, the characterization of the fish community, guppy risks of predation and the multifaceted approach to quantifying colour patterns. Significant innovations have arisen since, including techniques to better characterize structural colour [14, 23, 24], greater insight into the visual capacities of guppies and their predators [8, 12], and an increased understanding of how guppies behave in the wild [46]. Likewise, the continued development of tools for assessing colour patterns has delivered innovations such as QCPA [29] and colourmesh [16] that provide composite assessments of complex phenotypes. In this paper we raise concerns aimed not at the new analytical tools themselves but the way they were applied [15, 16]. These papers demand close scrutiny because, despite using substantially different methodologies for assessing male colouration, they each strongly conclude that their results nullify Endler's existing paradigm rather than asking why there are differences.

Consideration of the five points outlined above offers manifold reasons why the studies of Yong *et al.* and Valvo *et al.* neither dispute Endler's original results nor reasonably address the hypothesis of parallel evolution. Both studies rely upon a biased characterization of colour pattern that ignores the importance of structural colour in long-range detection by predators [2] and variation among localities in how such colours are selected by females [32, 35]. Neither study adequately addresses the composition of predator communities. Given the passage of time and the known changes in habitats [66], it is reasonable to expect significant investment to assess predator communities if one is to fairly appraise the generality of Endler's results. Further, exclusively focusing on colour patch boundaries (as in Yong *et al.*) is likely to misjudge the manner in which predation operates on colour patterns.

More broadly, guppy colour patterns comprise multi-trait phenotypes which will evolve according to multivariate selection in ways prescribed by genetic architecture. This generates complexity and constraints in how the colour pattern as a whole may evolve, even under comparable intensities of selection, and has profound consequences for what we should expect to constitute evidence for parallel evolution. For example, female guppies favour different colour traits in different ways [32, 35] which is likely to generate significant within-environment divergence. Expectations for evolutionary parallelism among populations subject to relaxed predation risk should not be for male colour patterns to evolve in the same ways, but according to the vectors initially favoured by females in each population. Studies of parallel evolution frequently find considerable variation within a selective regime [36, 47] and there are various degrees of parallelism, which means that treating it as a yes/no conclusion is unrealistic [47].

Finally, there remains a significant challenge to understand how the varied methods for quantifying colour patterns actually predict how such patterns are detected and appraised by ecologically-relevant viewing audiences. Endler recognized the potential limitations to atomizing male colouration into discrete components, which is why he suggested boundary analysis, which was expanded in QCPA (29) and used by [15]. In terms of biological relevance, however, it remains to be demonstrated that an arbitrarily holistic assessment of colour pattern is actually the best way to proceed, especially (as in [16]) if it has no known relationship to neurophysiology and perception. If particular components of a pattern serve critical roles in deciding mate attractiveness (such as tail colouration, for example [40]), or in determining visibility to predators, then analyses which attempt to account for the manifold differences among entire patterns may dilute analytical power and miss what is in fact important. Further, more complex and integrative appraisals of the nature provided by [16] can tell us if two groups of males are different from each other, but the analysis does not tell us about exactly how or why they differ, including whether and how one group may be more conspicuously coloured than the other.

The collective efforts to understand how and why male colouration has evolved in guppies can be considered emblematic of the challenges associated with studying colour evolution more generally (Table 1). Quantifying colour parameters isolation from biology is a necessary step in an otherwise much more complex process. One must consider the diverse ways in which colouration is generated and employ the diverse methods required to measure them appropriately. One must then consider the context in which colour is perceived, the visual sensitivity of the observer, and differences in what colour pattern traits are important to predators, potential mates, rivals and competitors (Table 1). We must also consider the differences between natural and sexual selection and how they might influence the repeatability of adaptive response. We look forward to continued research in systems such as guppies that allow insights into the full complexity of colour pattern evolution. Many of the principles outlined here will apply to other sensory modes.

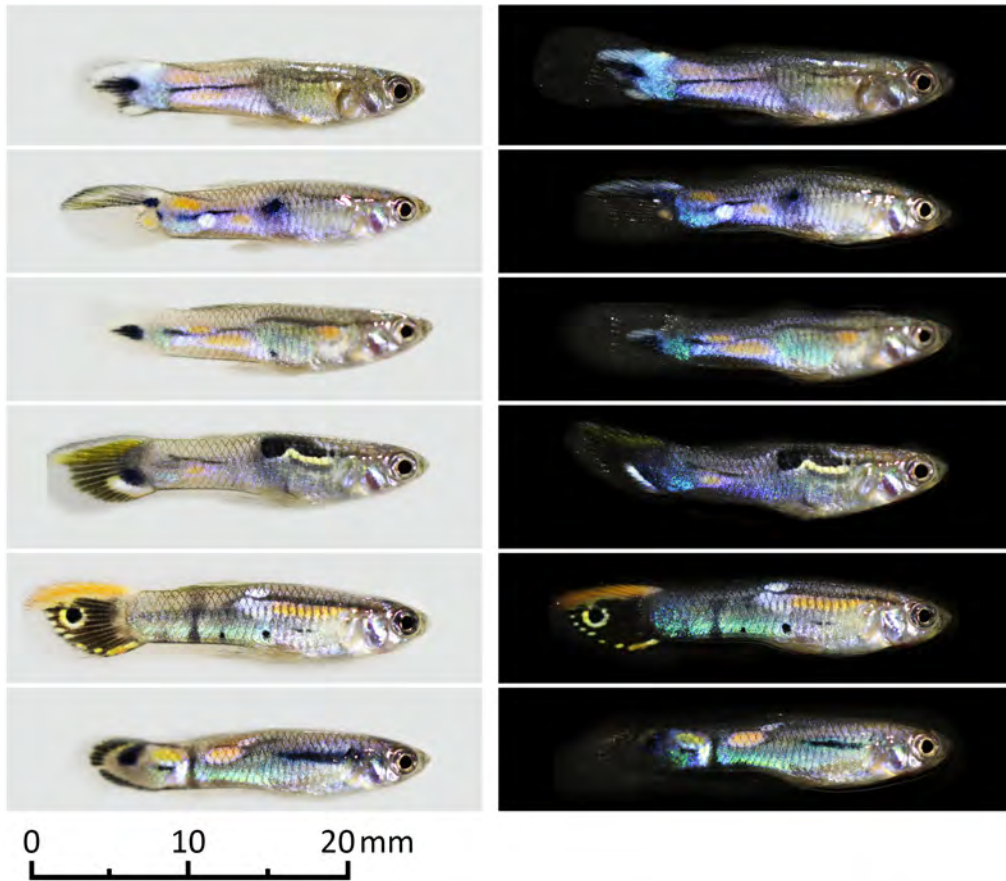


Figure 1: Six male guppies photographed against light and dark backgrounds. Males in the wild are likely to be viewed against both background types [46] and all gradations in-between (and frequently against light/dark mosaic backgrounds). Each background type enhances different features of the colouration: light backgrounds accentuate pigment-based orange and black markings, whereas dark backgrounds accentuate structural colouration. Biases against each background are especially prevalent across the caudal peduncle because the tissue substrate becomes more transparent towards the posterior of the fish. The caudal fin membrane is entirely transparent unless pigmented, which demands a light background to adequately display colour. These guppies are laboratory-reared descendants of wild females sampled from the Trinidad Guanapo drainage and were photographed as described in [14].

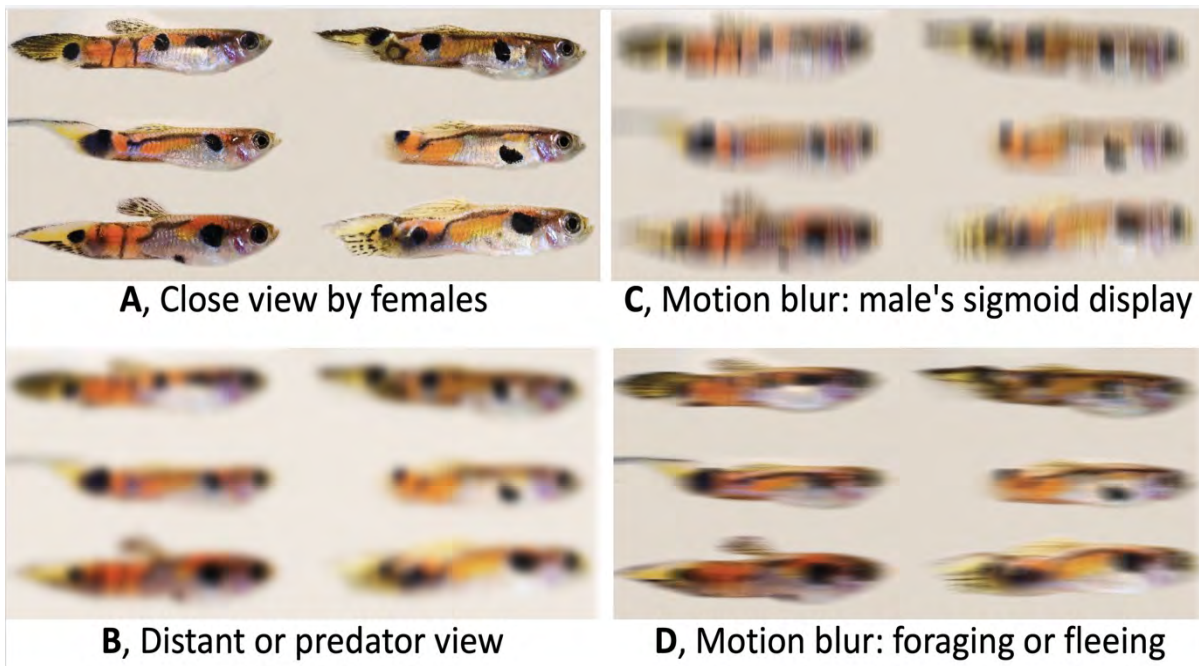


Figure 2: Blurring effects of acuity and motion (methods in online supplement). Structural colours show weakly in this photograph; see Figures S1 and S2 for effects on structural colours. **A:** Six guppies seen at short distance (as in typical guppy courtship). **B:** the same guppies seen at a distance (as by a predator). **C:** Motion blur during the sigmoid courtship display in which the body is rapidly rotated along the body long axis; blurring vertically. This also has a strong effect on the structural colours because they only reflect strongly in certain directions, causing a strong flicker. **D:** Motion blur when foraging or fleeing a predator; blurring horizontally. This kind of motion results in a lot less structural colour flicker because the long axis is not rotated very much, if at all. Note the loss of colour and luminance contrast due to acuity limits and note the very different appearances due to motion in different directions. Similar effects occur for structural colours (Figures S1 and S2). Guppies and their predators gather very different visual information.

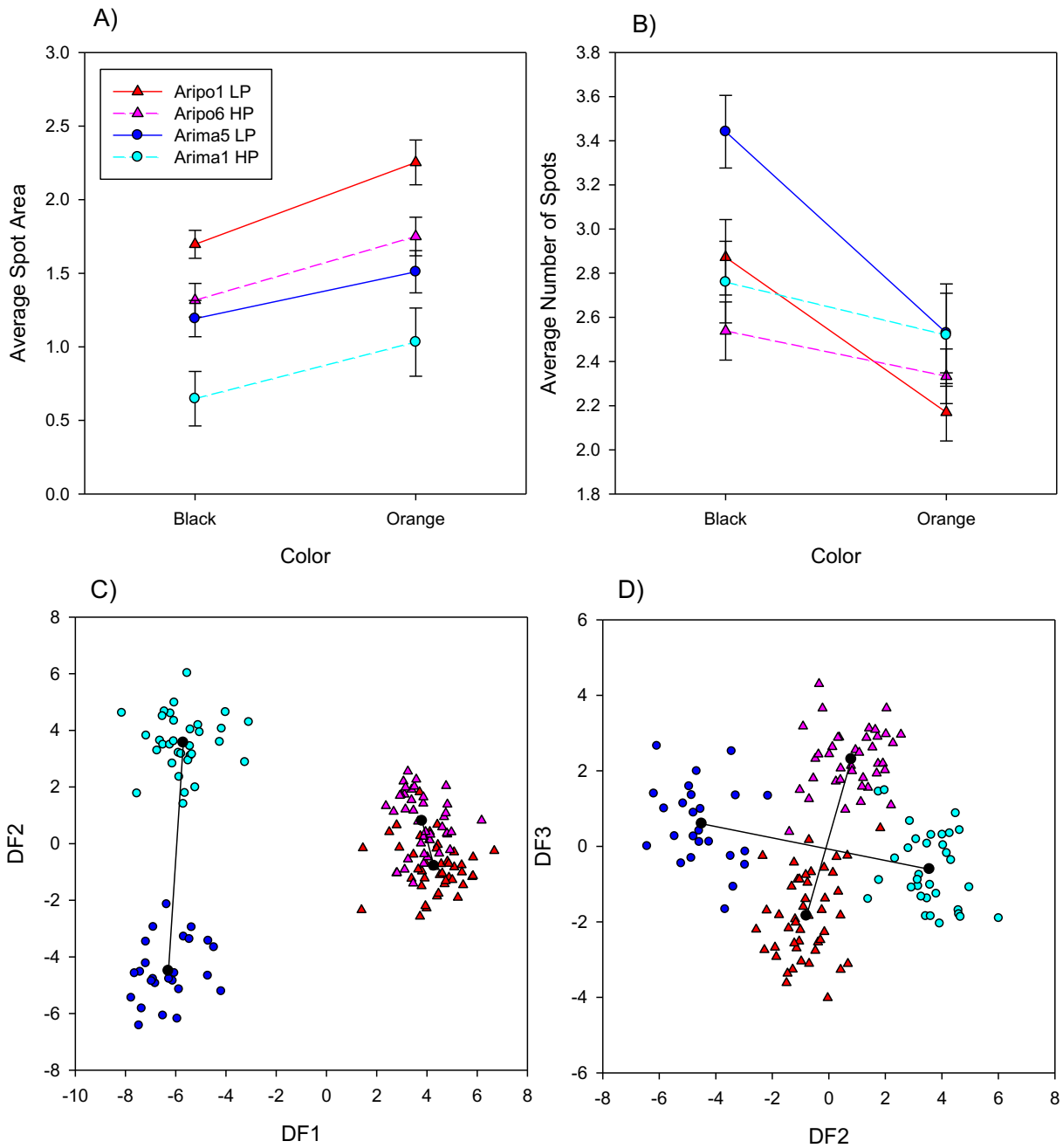


Figure 3: Analysis of a high-predation and low-predation population each from Aripo and Arima rivers. **A:** average (mm, ± 1 s.e.) black and orange spot area. **B:** average (± 1 SE) spot number. Low-predation populations had larger spots of both colours and more black spots ($p < 0.001$ for each) in both rivers but neither showed a difference in orange spot number ($p = 0.09$). We also conducted a DAPC on colourmesh data on the same fish. **C:** DF1 and DF2 with DF1 differentiating among rivers and DF2 between Arima predation regimes. **D:** DF2 and DF3, the latter differentiates among predation regime in the Aripo river.

Table 1, Important considerations for analysis and conclusions based upon colouration

Each heading is a process which depends upon the items in the list. The last line or lines provide some consequences. Further details in [4, 6, 55].

Ambient light spectrum and intensity

- season and time of day
- relative amounts of sunlight and clouds
- canopy closure, cloud cover and clouds over sun or not
- affects all aspects of vision

Reflection off both the average and current visual background

- what is in the field of view
- ambient light spectrum on background
- spatial variation within the background
- affects chromatic and luminance light adaptation hence colouration perception

Reflection off the guppy (or other target animal)

- ambient light striking the guppy
- reflectance spectrum from each colour pattern component
- the angle of structural colours relative to ambient light and viewer
- whether light is direct or diffuse; depends upon cloud cover
- spectral attenuation by the water between the guppy and viewer (distance dependent)
- with ambient light, determines spectrum, intensity & direction of guppy-viewer radiance
- affects reception and perception of colour patterns

Phenology of viewer and viewed

- time of day and microhabitat during which courtship or social activities occur
- time of day and microhabitats during maximum and average predation risk
- light spectrum and intensity changes with time of day and weather directly
- affecting visibility of colour patterns

Reception by viewer

- viewer's attention captured by the guppy (viewing correct direction)
- ambient light striking the guppy
- distance and angle to guppy
- water attenuation
- relative spectra of guppy and visual background
- relative complexity of guppy and visual background
- spectral composition of light coming from viewers field of view and guppy
- the resolving power (acuity) of viewer
- the viewer's flicker fusion rate (ability to see moving objects)
- the guppy's speed in the viewer's field of view
- the geometry of the guppy's motion
- the within-guppy visual contrast
- the guppy-background visual contrast (dynamic)
- affects what reaches the viewer's eyes
- viewer species can vary extensively in visual and other sensory abilities

Retinal processing and detection by the viewer

- spectral sensitivity of viewer's photoreceptors
- relative abundance of viewer's photoreceptor classes
- chromatic adaptation of viewer's photoreceptors
- the photoreceptors reaction times (photopigment regeneration rate) of viewer
- visual neural circuitry
- susceptibility to sensory biases
- spatial and temporal patterns of variation in retinal processing resulting from
 - variation in environment, background and motion of both viewer and viewed
- different for each viewer, even within species

Perception and Discrimination by the viewer

viewer's attention captured by the guppy
cognitive processes including colour classification and categorization,
use to which colour pattern information is put, previous experience with colouration, etc.
within-guppy and guppy-background visual contrasts and their relative values
--likely to vary among viewers, both among and within species.
--likely to vary within a viewer, depending upon viewer's physiological state & experience

Preferences and decision making by the viewer

viewer's attention held long enough to make a decision about the guppy
viewer's inherent preferences, usual use of colour patterns & colour-based decisions
current and previous experience with consequences of choices based upon colouration
--likely to vary among viewers, both among and within species.
--likely to vary within a viewer, depending upon viewer's physiological state & experience

Guppy (or other prey) fitness consequences

which predator species are present and which species are most risky
local mean and variation in female preferences
environmental conditions including environmental changes at all time scales
--can be modified by guppy behaviour such as microhabitat choice and phenology

References

1. Nagel, T. (1974) What is it like to be a bat? *The Philosophical Review* 83, 435-450.
2. Endler, J.A. (1978) A predator's view of animal color patterns. *Evolutionary Biology* 11, 319-364.
3. Endler, J.A. (1990) On the measurement and classification of colour in studies of animal color patterns. *Biological Journal of the Linnean Society* 41 (4), 315-352.
4. Kemp, D.J. et al. (2015) An integrative framework for the appraisal of coloration in nature. *American Naturalist* 185 (6), 705-724.
5. Endler, J.A. and Mielke, P.W. (2005) Comparing entire colour patterns as birds see them. *Biological Journal of the Linnean Society* 86 (4), 405-431.
6. Endler, J.A. and Mappes, J. (2017) The current and future state of animal coloration research. *Philosophical Transactions of the Royal Society of London (B)* 372 (1724), 8.
7. Endler, J.A. (1987) Predation, light intensity and courtship behaviour in *Poecilia reticulata* (Pisces: Poeciliidae). *Animal Behaviour* 35, 1376-1385.
8. Sandkam, B. et al. (2018) Reviewing guppy color vision: integrating the molecular and physiological variation in visual tuning of a classic system for sensory drive. *Current Zoology* 64 (4), 535-545.
9. Grether, G.F. et al. (2001) Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual colouration in guppies (*Poecilia reticulata*). *Proceedings of the Royal Society of London (B)* 268, 1245-1253.
10. Grether, G.F. et al. (2004) Individual colour patches as multicomponent signals. *Biological Reviews* 79 (3), 583-610.
11. Endler, J.A. (1991) Variation in the appearance of guppy color patterns to guppies and their predators under different visual conditions. *Vision Research* 31 (3), 587-608.
12. Weadick, C.J. et al. (2012) Visual pigment molecular evolution in the Trinidadian pike Cichlid (*Crenicichla frenata*): A less colorful world for neotropical cichlids? *Molecular Biology and Evolution* 29 (10), 3045-3060.
13. Houde, A.E. (1997) Sex, Color and Mate Choice in Guppies, Princeton University Press.
14. Kemp, D.J. et al. (2018) Predictable adaptive trajectories of sexual coloration in the wild: evidence from replicate experimental guppy populations. *Evolution* 72 (11), 2462-2477.
15. Yong, L. et al. (2021) Sensory-based quantification of male colour patterns in Trinidadian guppies reveals no support for parallel phenotypic evolution in multivariate trait space. *Molecular Ecology* 31 (5), 1337-1357.
16. Valvo, J.J. et al. (2021) Using Delaunay triangulation to sample whole-specimen color from digital images. *Ecology and Evolution* 11, 12468-12484.
17. Haskins, C.P. et al. (1961) Polymorphism and population structure in *Lebistes reticulatus*, a population study. In *Vertebrate Speciation* (Blair, W.F. ed), pp. 320-395, University of Texas Press.
18. Haskins, C.P. et al. (1970) Stabilized heterozygosis of supergenes mediating Y-linked color patterns in *Lebistes reticulatus*. *Heredity* 25, 575-589.
19. Baerends, G.P. et al. (1955) Ethological studies on *Lebistes reticulatus* (Peters). I. An analysis of the male courtship pattern. *Behaviour* 8 (4), 249-334.
20. Kodric-Brown, A. and Johnson, S.C. (2002) Ultraviolet reflectance patterns of male guppies enhance their attractiveness to females. *Animal Behaviour* 63, 391-396.
21. Smith, E.J. et al. (2002) Ultraviolet vision and mate choice in the guppy (*Poecilia reticulata*). *Behavioral Ecology* 13 (1), 11-19.
22. Millar, N.P. and Hendry, A.P. (2012) Population divergence of private and non-private signals in wild guppies. *Environmental Biology of Fishes* 94, 513-525.
23. Kemp, D.J. et al. (2008) Ornamental evolution in Trinidadian guppies (*Poecilia reticulata*): insights from sensory processing-based analyses of entire colour patterns. *Biological Journal of the Linnean Society* 95 (4), 734-747.
24. Kemp, D.J. et al. (2009) Predicting the direction of ornament evolution in Trinidadian guppies (*Poecilia reticulata*). *Proceedings of the Royal Society of London (B)* 276 (1677), 4335-4343.
25. Houde, A.E. (1987) Mate choice based upon naturally occurring color-pattern variation in a guppy population. *Evolution* 41 (1), 1-10.
26. Godin, J.G.J. and McDonough, H.E. (2003) Predator preference for brightly colored males in the guppy: a viability cost for a sexually selected trait. *Behavioral Ecology* 14 (2), 194-200.
27. Endler, J.A. (1980) Natural selection on color patterns in *Poecilia reticulata*. *Evolution* 34 (1), 76-91.
28. Endler, J.A. (1983) Natural and sexual selection on color patterns in poeciliid fishes. *Environmental Biology of Fishes* 9 (2), 173-190.

29. van den Berg, C. et al. (2020) Quantitative Colour Pattern Analysis (QCPA): A comprehensive framework for the analysis of colour patterns in nature. *Methods in Ecology and Evolution* 11 (2), 316-332.
30. Walsh, B. and Blows, M.W. (2009) Abundant genetic variation plus strong selection = multivariate genetic constraints: a geometric view of adaptation. *Annual Review of Ecology Evolution and Systematics* 40, 41-59.
31. Roff, D.A. and Fairbairn, D.J. (2012) A test of the hypothesis that correlational selection generates genetic correlations. *Evolution* 66 (9), 2953-2960.
32. Endler, J.A. and Houde, A.E. (1995) Geographic variation in female preferences for male traits in *Poecilia reticulata*. *Evolution* 49 (3), 456-468.
33. Brooks, R. and Endler, J.A. (2001) Female guppies agree to differ: phenotypic and genetic variation in mate-choice behavior and the consequences for sexual selection. *Evolution* 55 (8), 1644-1655.
34. Simmons, L.W. et al. (2001) Geographic variation in female preference functions and male songs of the field cricket *Teleogryllus oceanicus*. *Evolution* 55 (7), 1386-1394.
35. Houde, A.E. and Endler, J.A. (1990) Correlated evolution of female mating preferences and male color patterns in the guppy *Poecilia reticulata*. *Science* 248 (4961), 1405-1408.
36. Kranz, A.M. et al. (2018) Colour pattern component phenotypic divergence can be predicted by the light environment. *Journal of Evolutionary Biology* 31 (10), 1459-1476.
37. Fisher, R.A. (1930) *The Genetical Theory of Natural Selection*, 2 edn., Clarendon Press.
38. Millar, N.P. et al. (2006) Disentangling the selective factors that act on male colour in wild guppies. *Oikos* 113 (1), 1-12.
39. Lindholm, A.K. et al. (2014) Causes of male sexual trait divergence in introduced populations of guppies. *Journal of Evolutionary Biology* 27 (2), 437-448.
40. Hughes, K.A. et al. (1999) Familiarity leads to female mate preference for novel males in the guppy, *Poecilia reticulata*. *Animal Behaviour* 58, 907-916.
41. Hughes, K.A. et al. (2013) Mating advantage for rare males in wild guppy populations. *Nature* 503, 108-110.
42. Valvo, J.J. et al. (2019) Consistent female preference for rare and unfamiliar male color patterns in wild guppy populations. *Behavioral Ecology* 30 (6), 1672-1681.
43. Farr, J.A. (1977) Male rarity or novelty, female choice behavior, and sexual selection of the guppy, *Poecilia reticulata* (Pisces: Poeciliidae). *Evolution* 31, 162-168.
44. Houde, A.E. (1988) Genetic difference in female choice between two guppy populations. *Animal Behaviour* 36, 510-516.
45. Fleishman, L.J. et al. (2020) The interacting effects of total light intensity and chromatic contrast on visual signal visibility in an *Anolis* lizard. *Animal Behaviour* 167, 263-273.
46. Endler, J.A. et al. (2022) Visual background choice and light environment affect male guppy visual contrast. *Vision* 6 (3), 56.
47. Bolnick, D.I. et al. (2018) (Non)Parallel evolution. *Annual Review of Ecology, Evolution, and Systematics*, Vol 49 49, 303-330.
48. Caves, E.M. et al. (2018) Visual acuity and the evolution of signals. *Trends in Ecology & Evolution* 33 (5), 358-372.
49. Land, M.F. (2019) Eye movements in man and other animals. *Vision Research* 162, 1-7.
50. Titcomb, G.C. et al. (2014) Evaluating scope for flicker-fusion as a defensive strategy in coral snake mimics. *Current Zoology* 60, 123-130.
51. Sibeaux, A. et al. (2021) The role of boundary length and adjacent patch contrast in guppy mate choice. *Behavioral Ecology* 32 (1), 30-40.
52. van den Berg, C.P. et al. (2022) Colour pattern edge contrast statistics can predict detection speed and success at ecologically relevant viewing distances in triggerfish (*Rhinecanthus aculeatus*). *bioRxiv*.
53. Troscianko, J. et al. (2017) Quantifying camouflage: how to predict detectability from appearance. *Bmc Evolutionary Biology* 17.
54. Vukusic, P. et al. (1999) Quantified interference and diffraction in single *Morpho* butterfly scales. *Proceedings of the Royal Society of London (B)* 266, 1403-1411.
55. Lythgoe, J.N. (1979) *The Ecology of Vision*, Oxford University Press.
56. Hasson, O. (1991) Sexual displays as amplifiers: practical examples with an emphasis on feather decorations. *Behavioral Ecology* 2, 189-197.
57. Shawkey, M.D. and Hill, G.E. (2005) Carotenoids need structural colours to shine. *Biology Letters* 1, 121-124.
58. Rutowski, R.L. et al. (2005) Pterin pigments amplify iridescent ultraviolet signal in males of the orange sulphur butterfly, *Colias eurytheme*. *Proceedings of the Royal Society of London (B)* 272 (1578), 2329-2335.
59. Echeverri, S.A. et al. (2021) How signaling geometry shapes the efficacy and evolution of animal communication systems. *Integrative and Comparative Biology* 61 (3), 787-813.

60. Reznick, D. and Endler, J.A. (1982) The impact of predation on life-history evolution in Trinidadian guppies (*Poecilia reticulata*). *Evolution* 36 (1), 160-177.
61. Seghers, B.H., An analysis of geographic variation in the antipredator adaptations of the guppy, *Poecilia reticulata*, University of British Columbia, Vancouver, British Columbia, Canada., 1973.
62. Fraser, D.F. and Gilliam, J.F. (1992) Nonlethal impacts of predator invasion: facultative suppression of growth and reproduction. *Ecology* 73, 959-970.
63. Gilliam, J.F. et al. (1993) Structure of a tropical fish community: a role for biotic interactions. *Ecology* 74, 1856-1870.
64. Torres-Dowdall, J. et al. (2012) Local adaptation and the evolution of phenotypic plasticity in Trinidadian guppies (*Poecilia reticulata*). *Evolution* 66 (11), 3432-3443.
65. Endler, J.A., Freshwater fishes and crustaceans of the Northern Range, Trinidad., James Cook University Archive. <https://doi.org/10.25903/518b-hb05>, 2022.
66. Ehlman, S.M. et al. (2020) Altered visual environment affects a tropical freshwater fish assemblage through impacts on predator-prey interactions. *Freshwater Biology* 65 (2), 316-324.
67. Felmy, A. et al. (2022) Life histories as mosaics: Plastic and genetic components differ among traits that underpin life-history strategies. *Evolution* 76 (3), 585-604.
68. Reynolds, R.J. et al. (2010) Multiyear study of multivariate linear and nonlinear phenotypic selection on floral traits of hummingbird-pollinated *Silene virginica*. *Evolution* 64 (2), 358-369.
69. De Lisle, S.P. and Bolnick, D.I. (2020) A multivariate view of parallel evolution. *Evolution* 74 (7), 1466-1481.

Supplement to

How to generate and test hypotheses about colour: insights from half a century of guppy research

Darrell Kemp, David Reznick, Jeffrey Arendt, Cedric van den Berg and John A. Endler

This supplement provides additional images of the effects of defocusing and motion blurring of guppy colour patterns. It shows the effects on white and black backgrounds in order to show the effects on structural as well as pigment-based colours.

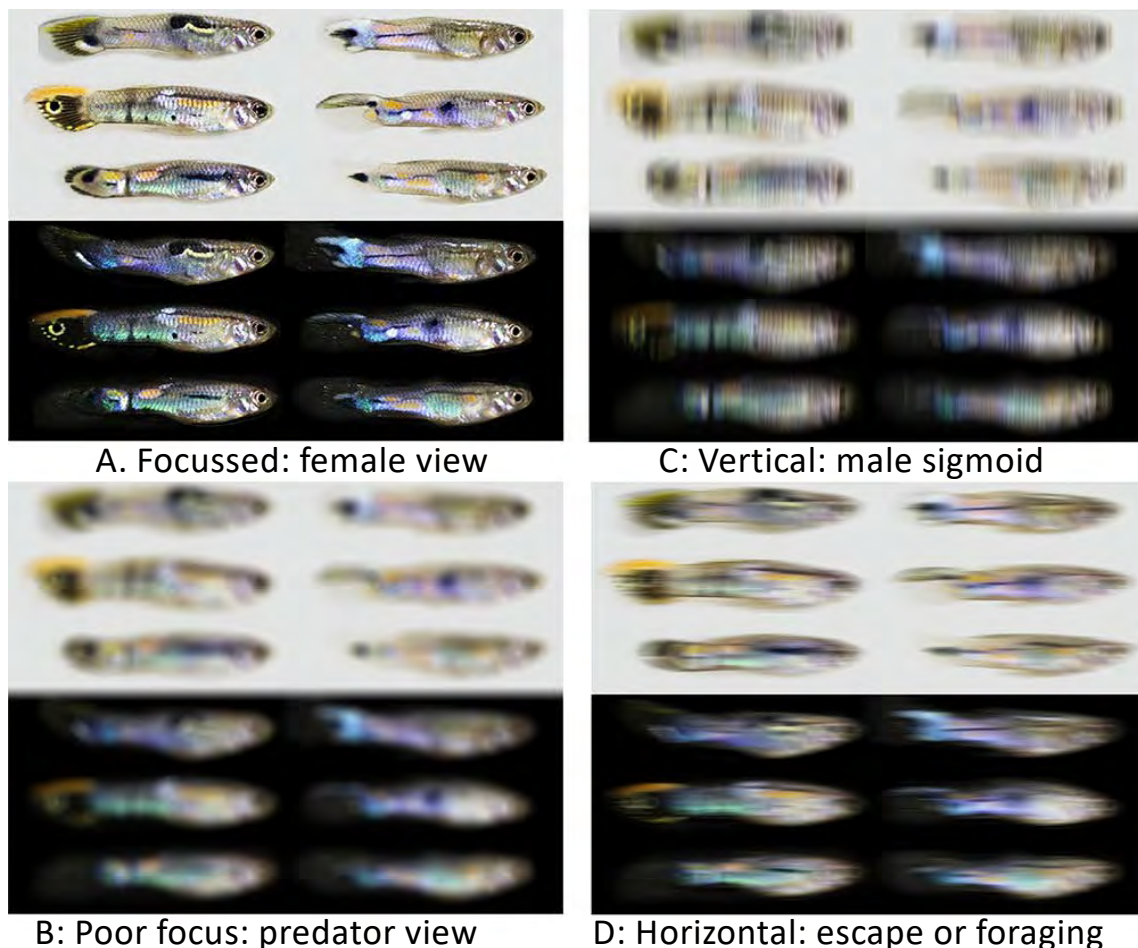
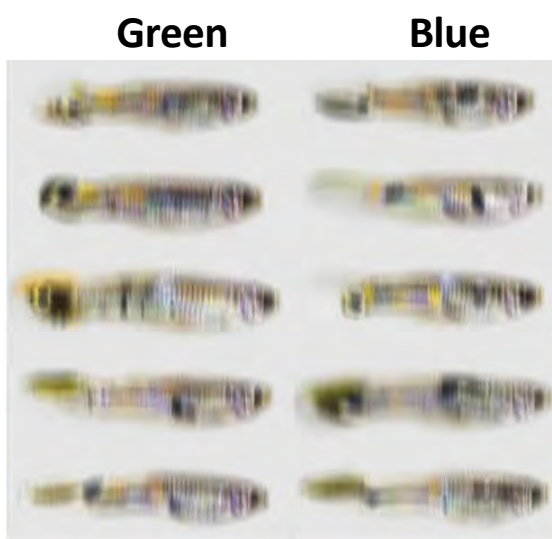
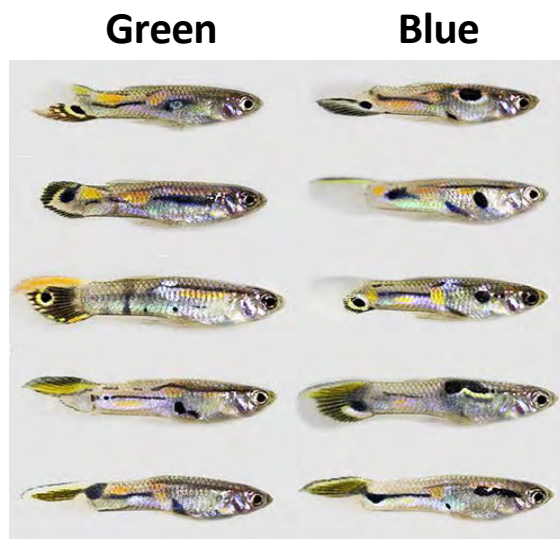


Figure S1. As in Figure 2, there are 4 groups of images: (A) focussed—as the female sees males up close; (B) poor focus—as predators see guppies from a distance; (C) Vertical—vertical motion blur during the male's sigmoid display to the female; (D) Horizontal—horizontal blur when guppy is escaping or foraging. Images of the same fish against white and black backgrounds as in Figure 1.

White background



Black background

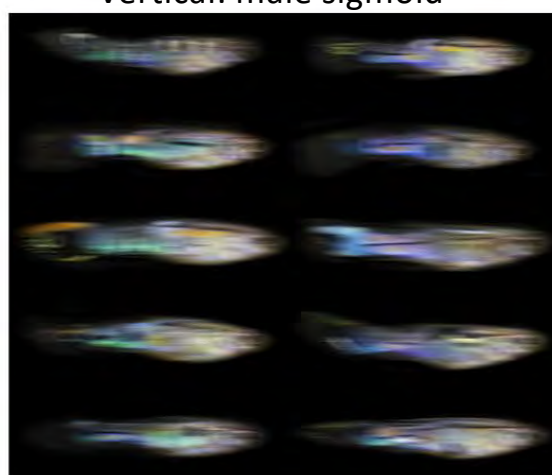
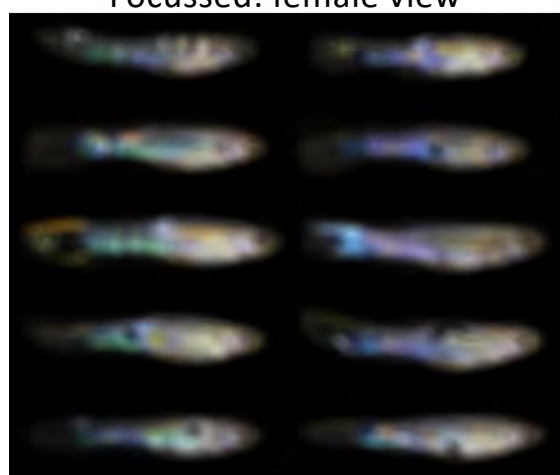
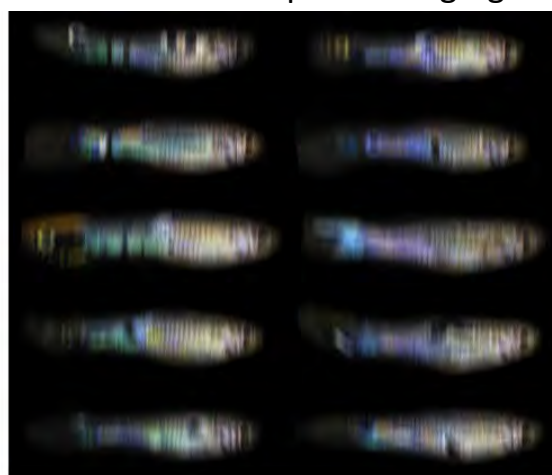
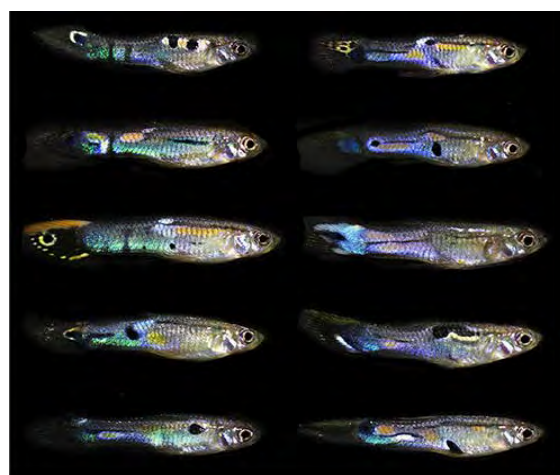


Figure S2. Effects of defocus and motion blur on a diversity of guppy colour patterns. *Within panels* the fish in the left columns were chosen to emphasize green structural colours and those on the right columns chosen to emphasize blue structural colours under optimal lighting angles. As a result, and unlike figure S1, only some fish appear on both white and black backgrounds

MATLAB programme that will blur or motion blur any image.

The programme reads the image, then does a simple defocus, saves the new image to a file, then does the horizontal and then the vertical blurring. Images are saved as .png at 300 dpi but you can save as other formats and resolutions.

`fspecial` creates the filter which determines how the image is processed.

`imfilter` uses the filter to modify the image.

MATLAB has detailed help on how to use these (and other) functions.

Guppies.jpg is the name of the image used in figure 2 of the main paper.

Note that this figure size is 982 x 2306 pixels;

figures with different scales will require different filter parameters

```
clear;
```

```
fn='Guppies.jpg'; img=imread(fn); imshow(img);
```

```
h=fspecial('disk',40); %defocus scale 40 pixels
```

```
dfoc=imfilter(img,h,'replicate');
```

```
figure; set(gcf,'Position',[815 467 924 547]);
```

```
imshow(dfoc); title('defocus 40');
```

```
print('Defocussed.png','-dpng','-r300');
```

```
h=fspecial('motion',100,0); %100 pixels horizontal
```

```
hor=imfilter(img,h,'replicate');
```

```
figure; set(gcf,'Position',[815 467 924 547]);
```

```
imshow(hor); title('horizontal 100 p');
```

```
print('Horizontal.png','-dpng','-r300');
```

```
h=fspecial('motion',100,90); %100 pixels vertical
```

```
vert=imfilter(img,h,'replicate');
```

```
figure; set(gcf,'Position',[815 467 924 547]);
```

```
imshow(vert); title('vertical 100 p');
```

```
print('Vertical.png','-dpng','-r300');
```