Facial Coloration as a Social Signal in a Neotropical Cichlid: Testing the Facial Expression Hypothesis

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Abstract

The face is a uniquely distinctive stimulus for humans, encapsulating a wealth of information. Among the myriad of social cues conveyed by the face, emotional signals, known as facial expressions, are paramount not only for humans but also for numerous social animals. The evolution of facial expressions in these animals can also manifest in taxa other than mammals, as suggested by various studies highlighting the socio-ecological benefits of facial expressions. In this study, we elucidated the social function of facial coloration, determined by melanophores, in the neotropical social cichlid *Symphysodon aequifasciatus*. In this species, facial coloration exhibits flexibility and instantaneous changes in response to varying social contexts. Through behavioral observations and experimental manipulation, we confirmed that facial coloration in *S. aequifasciatus* serves to attenuate unnecessary aggressive competition among conspecifics. Furthermore, we observed that the facial area subjected to coloration in this species is innervated by the adenosine triphosphate- and noradrenaline-ergic nervous system. These findings indicated that facial expression in *S. aequifasciatus* depends on the sympathetic nervous system and has evolved independently of mammalian facial expressions. Our study highlights teleost fishes as valuable animal models for exploring the universality of facial expressions and their underlying cognitive mechanisms in vertebrates.

Introduction

Human faces, which are the most important stimuli in various social contexts, are remarkably unique as they encapsulate a large amount of information and social cues related to various aspects, including sex [1], identity [2], emotional states [3], and attentive states [4,5]. In particular, humans are capable of manifesting emotions through facial movements (commonly referred to as facial expressions), which comprise specific motions that support optimal emotional signaling and are controlled by specific neural systems [6]. Human expressions are primarily produced by the action of muscles below the facial skin, commonly known as facial muscles [6,7]. These muscles are innervated by the facial nerve, which has the largest motor nucleus among all motor nuclei in the brainstem [8]. Additionally, the involvement of at least five cortical regions in the upstream regulation of motor control highlights the crucial role of facial expressions in humans [8]. Facial expressions are appropriately interpreted by the receiver's specialized neural circuits, including regions such as the superior temporal sulcus and amygdala, thereby conveying complex social and emotional information [9-12]. These elaborate systems are universal among humans, irrespective of cultural differences [13]. It is undeniable that facial expressions hold a unique and significant role in the communication of human emotions. Thus, delving into the evolutionary history of facial expressions presents an intriguing challenge within the realm of evolutionary biology.

A valuable methodology for elucidating the evolutionary trajectory of human facial expressions involves juxtaposing them with patterns of emotional expression observed in animals. It was Charles Darwin who initially recognized the significance of comparing the facial expressions of animals and humans, along with their associated cognitive abilities [14, 15]. In his seminal work "*The Expression of the Emotions in Man and Animals*," Darwin postulated that human emotional expressions have evolved from those of our animal ancestors rather than being the product of our society, suggesting their comparability [16], a proposition that sparked

considerable controversy [14]. While the degree of comparability between human and animal facial expressions remains a contentious subject, research in the 21st century has provided evidence that non-human primates are able to convey many of the same facial expressions observed in humans through intricate facial muscle movements [17,18]. These studies suggest that facial expressions in primates are species-specific and that some are socially learned, while also reporting many innate aspects [19]. However, regardless of the patterns of facial expression in primates, the dominant theory is that the face is important as a signal for the communication of emotional states to other individuals. Moreover, there has been some debate as to what extent the use of specialized visual cues from the face and the consequent development of associated specializations within the neural system are unique features found only in primates. For instance, laboratory mice [20] and macaws [21] have been shown to display a variety of discernible facial expressions.

Interestingly, as observed in humans, faces in these animals are recognized as vehicles for conveying information and social cues related for example to identity, dominance rank, and sex [19,22], alongside emotional signals. In these animals, multiple social cues are thought to be centralized in the face because of the evolutionary consequences of attention to the eyes [19]. Moreover, eyes serve as important signals to gaze direction and objects of interest by other individuals [23,24], resulting in social benefits for both signalers and signal receivers in social animals. Importantly, according to these theories, we can predict that the centralization of social cues, including facial expressions and the cognitive systems that process them, occur convergently in social animals, regardless of the taxonomic group.

Social fishes are good candidates for testing this hypothesis regarding the evolution and social benefits of facial expression. Teleost fishes lack the elaborated facial musculature of mammals; therefore, it has been thought that emotional facial expressions are restricted in this taxon [19]. However, recent studies have pointed to peculiarities in the faces of social fishes [25].

Specifically, numerous species spanning various taxonomic groups have been documented to exhibit notable morphological or color variations localized to the face serving as social cues for inter-specific or individual recognition [25-31]. Moreover, it has been posited that fish possess specialized cognitive and neural processing mechanisms for facial recognition [28, 32] and that the facial area may also play a pivotal role in self-recognition in cleaner wrasse [33]. As well as using social signals related to individual recognition, social fishes are known to initially focus on the face when presented with images depicting conspecifics [30], like primates do [34]. All these investigations underscore the face-related peculiarities in fish. Studying facial expressions in these animals, the vertebrates situated at the furthest phylogenetic distance from mammals (which have traditionally been the primary subjects of such studies), contributes to a deeper understanding of the general principles underlying the evolution of this phenomenon. However, to date, no studies have been conducted on emotional signals in fish based on the perspective of facial expressions.

To address this knowledge gap, we here focused on the social function of facial coloration in the discus fish *Symphysodon aequifasciatus*, a neotropical cichlid species that dwells in the Amazon River basin [35]. These fish reside in sunken trees alongside their conspecifics during the non-breeding dry season and reproduce in flooded forests throughout the breeding rainy season [36]. Both male and female members in reproductive pairs provide parental care and nourishment in the form of mucus for their offspring [37-39]. Remarkably, *S. aequifasciatus* individuals are able to differentiate between their breeding partner and unfamiliar fish by analyzing the structural color pattern on their faces [25]. Moreover, we observed that the vertical black bar located above their eyes, which is composed of melanophores [40], undergoes frequent changes during social interactions with conspecifics (Fig. 1a; Supplemental movie S1). Individuals have vertical black bars on their body, including the facial area. Despite population and individual differences in vertical black bars along the body flank, facial black bars (the so-

called eye-patch pattern) are universal among discus fishes [41]. Therefore, we hypothesized that this facial coloration may serve as a mode of emotional expression.

In this study, we aimed to clarify whether facial coloration in *S. aequifasciatus* functions as an emotional signal by using two complementary approaches: behavioral experiments and neuroscientific methods. In particular, we evaluated whether immediate changes in facial coloration can be referred to as facial expression by testing 1) whether color change was concentrated on the face in different social contexts and 2) whether variation in face color suppressed unnecessary social costs. Through a combination of behavioral observations and experimental manipulation of facial coloration, we seeked to elucidate the role of this phenomenon as a social signal. Moreover, using experiments to determine which neurotransmitters activate melanophores in the facial area [40] and the slightly modified DEEP-Clear method [42], a clearing method for exploring the peripheral innervation of facial areas subjected to coloration, we attempted to identify which peripheral nerves are responsible for face color changes in *S. aequifasciatus* and examined whether these are analogous to facial expressions in mammals.

Results and Discussion

Association between social behaviors and facial pigmentation patterns

To explore the association between distinct social behaviors and pigmentation patterns, we conducted behavioral observations of free-ranging *S. aequifasciatus* individuals (n = 8 males and 4 females) under simulated natural conditions. The pigmentation pattern, which encompasses melanophores in discus fishes, was classified into three categories: 1) complete absence of all vertical bars on the body, 2) manifestation of solely the eye-patch pattern, and 3) manifestation of all vertical bars, including the eye-patch pattern (Fig. 1b). Specifically, when exhibiting aggressive behavior toward conspecifics, individuals typically abstained from

displaying eye-patch patterns (Fig. 1b). Conversely, when under attack or during periods of rest and foraging, they did show them. Interestingly, eye patch patterns disappeared during sexual behavior as well as during aggression. With regard to the third pigmentation pattern, in which all vertical bars are manifested, individuals did not show it frequently, regardless of social behaviors.

The pigmentation patterns in *S. aequifasciatus* were clearly correlated with certain social behaviors and were subject to rapid alteration, as shown in Supplementary movie S1. Numerous instances of body color change serving as a social signal have been documented in various social fishes. For example, in the group-living cichlid *Neolamprologus pulche*r [43] and mouth-brooding cichlid *Astatotilapia burtoni* [40,44], the intricate pigmentation pattern in the facial area serves as a social signal indicating aggressiveness, as similarly observed in discus fishes. However, there is no conclusive evidence that these changes in pigmentation pattern occur immediately in conjunction with the specific display, which would reflect a motive for aggression associated with social rank over a longer term, as suggested by Rodrigues et al. (2009) [45]. Color change in the facial area in discus fishes is more akin to facial expression reported in primates [19] than to variation in facial coloration reported in other fish species.

The expression of distinct emotional states on the human face is characterized by the subtle movements of facial musculature [13]. Conversely, in discus fishes, it is the alteration of pigmentation patterns in the facial area generated by the contraction of chromatophores, rather than facial muscles, that leads to the manifestation of specific facial expressions that correspond to specific emotional states. In particular, in this study, *S. aequifasciatus* individuals showed the eye-patch pattern when they were being fed or had received aggression but did not display it when engaged in aggressive or partner-specific behaviors. This suggested that the presence of the eye-patch pattern may be indicative of normalcy (submissive behavior) while its absence may denote excitement (aggressive behavior).

Benefits of facial coloration patterns as social signals

To examine whether specific facial coloration patterns influence the degree of same-sex sexual harassment perpetrated by dominant individuals, we observed a series of aggressive behaviors between subordinate and dominant individuals for 20 minutes. We specifically focused on whether a subordinate that was under attack could decrease the possibility of being subjected to continuous harassment (defined as an attack occurring within 5 seconds of the first attack) from a dominant individual by modifying its eye-patch pattern.

When a subordinate individual was attacked, alterations in facial coloration significantly affected the occurrence of persistent harassment (binomial generalized linear mixed models [GLMM], df = 3, $\chi^2 = 63.589$, $p < 0.001$) (Fig. 2). Subordinate individuals who did not exhibit the eye-patch pattern and did not modify their facial coloration upon being attacked by dominant individuals were significantly more likely to experience continuous harassment compared with those who did (df = 1, $\chi^2 = 54.186$, $p < 0.001$). Similarly, in subordinate individuals who maintained the eye-patch pattern before and during harassment, the possibility of continuous attacks also decreased (df = 1, $\chi^2 = 10.05$, $p = 0.002$). Conversely, the likelihood of continuous harassment remained comparable when subordinate individuals without the eye-patch pattern failed to alter their facial coloration during aggression, regardless of whether those initially displaying the pattern modified their facial coloration (df = 1, $\chi^2 = 2.093$, $p = 0.148$). Our behavioral observations suggested that alterations in facial coloration serve to deter harassment from dominant individuals in *S. aequifasciatus*.

However, because facial coloration in discus fishes may be correlated with a variety of social behaviors and psychological motivations [25], it is impossible to say whether it has a specific function in inhibiting harassment from dominant individuals. Therefore, we conducted experiments to manipulate facial coloration in *S. aequifasciatus* using anecdotal methods based on light exposure commonly employed by ornamental fish keepers. As shown in several studies conducted in the field of aquaculture science [46], exposing juvenile fish to intense light can affect

the melanophore-derived body coloration. Therefore, we obtained *S. aequifasciatus* individuals in which the function of melanophores was abolished by exposing them to intense light during the juvenile stage. The manipulated discus fish were unable to exhibit melanophore patterns regardless of social context (Supplementary S2), and thus were considered to be unable to convey proper social communication signals. In the behavioral experiment, we observed changes in aggressive interaction over time in experimental pairs in which 1) both individuals could properly display the eye-patch pattern (WW pairs, n = 8 pairs), 2) one individual was light-treated (WL pairs, n = 6 pairs), and 3) both individuals were light-treated (LL pairs, n = 7 pairs).

We detected a significant two-way interaction between the first day of the behavioral experiment and the type of experimental pair in terms of the frequency of aggressive interactions over a period of 20 minutes (negative binomial GLMM, df = 2, $\chi^2 = 67.968$, $p < 0.001$). In the WW group, the frequency of aggressive interactions between same-sex individuals decreased day by day, but in the LL group, which was composed of pairs in which both individuals who could not express optimal emotional signals, the frequency of aggressive interactions did not decrease within days (Fig. 3). In the LW group, the variation in the frequency of aggressive interaction was in-between the values observed for the WW and LL groups (Fig. 3). In this experiment, the manipulation inevitably affected not only facial coloration but also melanophore-derived pigmentation patterns in the rest of the body. However, considering that pigmentation patterns other than those in the face were largely unchanged in social displays between conspecifics (Fig. 1), the obtained results can be safely interpreted as being caused by the inability of facial melanophores to produce patterns, which therefore prevented proper communication between conspecifics.

During the experimental days, individuals lost 0.5%–8.4% of their body weight. The percentage of the reduction in body weight significantly varied among experimental groups, with LL suffering the greatest weight losses, followed by WL and WW, in that order (linear

227 mixed models [LMM], df = 2, F = 4.398, p = 0.025) (Fig. 4). Moreover, whether individuals
228 became dominant (or subordinate) during the experiment also had a marginal effect on body
229 weight reduction (df = 1, F = 3.955, p = 0.061). Sex (df = 1, F = 2.354, p = 0.145) and whether
230 individuals had been subjected to the light treatment (df = 1, F = 0.224, p = 0.641) had no
231 significant effects on the reduction. Social signals have been shown to confer benefits to both
232 conveyors and recipients [47]. The differential reduction in body weight observed across
233 experimental groups in this study, irrespective of social hierarchy, aligns with the tenets of
234 classical adaptive signaling theory and provides compelling evidence that facial coloration
235 yields socio-ecological benefits in *S. aequifasciatus*. This species forms social aggregates in the
236 Amazon River, an environment characterized by high predation pressure [36], and the act of
237 leaving the group, even to assume a subordinate position, carries fitness costs [48]. Here, the
238 individuals whose ability to modify facial coloration had been manipulated were possibly
239 unable to express the submissive signal toward dominant individuals and thus establish a
240 dominance hierarchy. Facial coloration may serve to mitigate superfluous competition within
241 social aggregates characterized by dominance hierarchies.
242
243 *Peripheral innervation regulating facial coloration*
244 The results of our behavioral experiments indicated that facial coloration in *S. aequifasciatus*
245 functions as a social signal in a similar manner to human facial expressions. One approach to
246 confirm whether the two phenomena are indeed analogous is to compare their underlying
247 regulatory mechanisms. Therefore, we conducted two experiments to elucidate the types of
248 peripheral nerves innervating the facial area subjected to changes in coloration in *S.*
249 *aequifasciatus*. Generally, discus fishes exhibit two primary mechanisms of color change:
250 physiological color change, mediated by hormonal and neural regulation of peripheral nerves,
251 and morphological color change, involving the accumulation or depletion of chromophores [49-51].
252 Previous behavioral experiments have shown that changes in facial coloration in discus fishes

occur rapidly, within a few seconds. Since morphological color changes usually require days to weeks [51], we hypothesized that the above-mentioned rapid changes are physiological, which are known to occur over shorter periods. In particular, they may be under neural control, which has been shown to regulate very rapid color changes within seconds [44, 49]. In discus fishes, body color changes, particularly those involving black coloration via the aggregation and dispersion of melanosomes within melanophores, are generally regulated by the sympathetic nervous system [44, 52, 53]. Sympathetic postganglionic neurons in discus fishes are known to possess several neurotransmitters, including noradrenaline (NA), adenosine triphosphate (ATP), neuropeptide Y, and acetylcholine [50, 54], the first two of which are the primary factors influencing the motile activity of melanosomes [49, 55]. Therefore, we first examined whether NA and ATP control facial coloration in discus fish by administering these neurotransmitters ex vivo. In this experiment, scales ($n = 20$) with chromatophores were freshly collected from eye patches and observed under a microscope. The scales were then immersed in neurotransmitters (1 μM NA or 10 mM ATP) or in PBS, which was used as a control. We quantified the rate of change in melanosome dispersion (%) in the scales and observed significant differences between ATP exposure and PBS exposure (two-way interaction between time and treatment, $df = 1$, $F = 200.888$, $p < 0.001$ by LMM), with melanosomes showing dispersion in the ATP exposure group (Fig. 5a, c). Conversely, NA exposure resulted in significant melanosome aggregation compared to PBS exposure (two-way interaction between time and treatment, $df = 1$, $F = 89.651$ $p < 0.001$) (Fig. 5b, d). These results suggested that changes in facial coloration in discus fish also depended on sympathetic innervation. To further validate this hypothesis, we investigated whether sympathetic postganglionic neurons (noradrenergic neurons) reached the eye-patch region via tissue clearing. Tissue clearing is a methodology to visualize appropriately labeled structures in chemically cleared, three-dimensional tissues, which is especially useful to track neurons or blood vessels [56, 57]. We cleared the heads of X individuals using the slightly modified DEEP-Clear method [42] and performed immunohistochemical staining using an anti-

tyrosine hydroxylase (TH) antibody (the key enzyme that converts tyrosine to L-DOPA, the initial step in NA biosynthesis). Finally, we performed refractive index matching using CUBIC-R reagent [58]. The results showed that TH-immunoreactive (TH-ir) fibers reached the eye-patch region (Fig. 5e). Furthermore, morphological observations indicated that these fibers connected to the scales containing the melanophores constituting the eye patch (Fig. 5f, g). These findings strongly suggested that sympathetic nerves innervate the facial area subjected to changes in coloration in discus fish.

Our results suggested that, unlike human facial expressions, which are regulated by facial nerves [7] via the neurotransmitter acetylcholine [59], facial coloration in the discus fish examined is regulated by the sympathetic nervous system. This has been similarly observed in the Tanganyikan cichlid, *Astatotilapia burtoni* [40,44]. Facial coloration serves as a social signal and plays a significant role in social interactions with other individuals [60, 61]. Therefore, its functioning to convey facial expression and its peripheral neural regulation observed in this study may not be limited to discus fish but could be prevalent among social fish species. One of the interesting aspects of our findings is that, while facial expressions and coloration similarly function as social signals in humans and fish respectively, they are controlled by different nervous systems. In humans, they are regulated by voluntary nerves, whereas in fish they are controlled by the autonomic nervous system. Regulation by voluntary nerves implies that humans can deceive others by displaying facial expressions that do not reflect their authentic emotions [62.63]. Recently, it has been reported that fish also possess the ability to deceive others tactically [64]. However, considering that facial coloration in fish is regulated by the autonomic nervous system, it is unlikely that they can use neurally controlled rapid variations in facial color for intentional deception. Conversely, fish employ various mechanisms for controlling body coloration beyond neural regulation, so deception using non-neural body color changes may still be possible [65].

In humans, blushing may function as a supplementary emotional signal that enhances facial expressions, though its impact is relatively less significant than that of facial expressions [66]. This phenomenon occurs involuntarily, triggered by sympathetic activation [66]. While the contexts and detailed mechanisms differ, blushing bears certain similarities to the coloration mechanism observed in discus fishes. Consequently, these findings may point to a form of convergent evolution with respect to the role of the sympathetic nervous system in mediating emotional expression through facial features.

Evolution of facial expression in social fishes

Of the body coloration composed of melanophores, *S. aequifasciatus* intensively linked and changed only facial coloration upon engaging in social behavior toward conspecifics. Our findings further validated that facial coloration in this species serves to mitigate superfluous aggressive competition among conspecifics, thereby benefiting both signalers and recipients within the dominance hierarchy. This supports the hypothesis that facial coloration in discus fishes exhibits functional parallels to mammalian facial expressions, predominantly those observed in primates [19]. Conversely, the neural mechanisms underlying facial coloration in these fishes markedly differ from those regulating facial expressions in mammals, strongly suggesting a convergent evolution of facial expressions in each lineage.

In humans, facial expressions are hypothesized to have initially evolved as a mechanism for modulating physiological states within an individual (e.g., widening of the eyes in expressions of fear, facilitating enhanced peripheral vision) before assuming a secondary role as social signals [67]. The vertical bars present on discus fishes, which are typically found among driftwood, may serve as forms of cryptic or disruptive coloration [68]. Indeed, when confronted with predators or subjected to prolonged intra-species aggression, discus fishes display vertical bars, including eye-patch patterns, spanning their entire body [41]. Similarly to facial expression in humans, facial coloration in discus fishes may have initially evolved as a cryptic form of

330 coloration mediated by melanophores, before subsequently being repurposed as a means to
331 convey submissive social signals localized solely to the facial region, undergoing
332 coevolutionary adaptations to facilitate their perception. Moreover, as an alternative prospective
333 evolutionary scenario other than cryptic coloration, the signals linked to long-term hierarchical
334 dominance cues within the facial region [40,43,44] may have undergone evolutionary divergence
335 toward immediate modification in response to social stimuli.

336 When a fish needs cryptic body coloration, for example when threatened by predators,
337 it experiences acute stress [69,70]. This stress activates the sympathetic nervous system, rapidly
338 increases systemic cortisol levels, and raises plasma glucose levels, creating a physiological
339 state conducive to fight-or-flight responses against the stressor [69-71]. A similar situation
340 assumably occurs when subordinate individuals try to avoid harassment from dominant
341 individuals [69,72-74]. Therefore, it should be plausible to hypothesize that the mechanism
342 regulating variations in body and facial coloration via the sympathetic nervous system to escape
343 from predator-related threats has secondarily adapted to cope with similar behavioral and
344 physiological contexts (for example to avoid harassment from dominant individuals), even
345 though the stressors are different.

346 However, facial coloration in discus fishes changes independently from body
347 coloration, suggesting the presence of distinct control mechanisms for these areas. It is
348 conceivable that discus fishes have developed neural mechanisms to gradually or alternatively
349 change the coloration of their face and body in response to different stimuli and social contexts.
350 Alternatively, differentiated neural pathways controlling facial and body coloration may have
351 evolved, as exemplified by the unique peripheral nerve branches found in these fish species,
352 such as the recurrent facial taste neurons in sea catfish, which have acquired taste buds on their
353 body surface and fins in response to socio-ecological demands over the course of evolution [75].
354 Although additional research is required, either scenario strongly supports the hypothesis that

social signals are centralized on the face in social animals, regardless of the mechanisms controlling facial and body coloration.

In recent years, various studies have examined the idiosyncrasies of faces to individual recognition [25,27-31], self-recognition [33], attention, and specialized cognitive functions in fish [28 32]. To the best of our knowledge, there have been no reports documenting instantaneous emotional signals on the faces of fish, as observed in discus fishes. However, this may stem from a paucity of research on facial expressions in this animal group rather than from an absence of such expressions. Indeed, in numerous fish species, social signals indicative of long-term dominance hierarchies are localized to the facial region [40,44,45], despite the face being an anatomically limited part of the body.

A comparative study conducted on primates has revealed a correlation between the efficacy of facial expression and social complexity [76]. Therefore, exploring the relationship between social systems and facial coloration in fish represents the next step to comprehensively elucidate the evolution of facial expression within this taxon. Our study demonstrates that teleost fishes are valid animal models for probing the universality of facial expressions and their underlying cognitive mechanisms in vertebrates.

Methods

Subjects and fish husbandry

Mature male and female *S. aequifasciatus* specimens were procured from ornamental fish traders that imported them from the Brasilia Amazon and bred F1 individuals in Japan. These specimens were housed in four separate water tanks. Uniform fish density and sex ratio were meticulously maintained across all stacked tanks. Water temperature and pH were carefully regulated and kept within the ranges of 27.0°C–30.0°C and 5.0–7.0, respectively. The fish were nourished with an artificial diet formulated from beef heart and mosquito larvae (three times per

380 day). The sex of specimens was determined based on their distinctive body coloration and
381 morphological characteristics. In total, eight males and four females were used for *behavioral*
382 *observation I*, and 20 males for *behavioral observation II*. All observations and experiments
383 were conducted from 12.00 to 15.00 and recorded using a video camera (HDR-CX470 and
384 HDR-CX370, Sony Corporation, Tokyo, Japan) placed at ca. 30 cm in front of the water tanks.
385
386 *Behavioral observation I: pigmentation patterns of S. aequifasciatus in different social contexts*

387 The behavior of *S. aequifasciatus* in a water tank was observed to determine what social context
388 correlated with pigmentation patterns, which change instantly (Supplemental movie S1).
389 Driftwood and dry leaves were placed in the tank (360 L, 120 × 60 × 50 cm), and the water was
390 colored using humic acid to mimic the natural habitat [36,41]. Three mature individuals (two males
391 and one female) were held in this tank for 3 days to allow accustomization to the environment.
392 During behavioral observations, which lasted for 25 min, mosquito larvae were fed to the fish to
393 promote social interaction among them, and the pigmentation patterns of focal fish were
394 evaluated when they showed six specific social behaviors (i.e., aggressive behavior, counter
395 aggression, received aggression, feeding, and partner display) based on the ethogram described
396 in Satoh et al. (2016)[25]. Aggressive behavior was defined as a fish approaching the lateral side
397 of a non-partner while opening its mouth and spreading its gill covers and fins as well as a fish
398 aggressively biting and/or bumping into the non-partner. A focal fish receiving aggressive
399 behaviors from other experimental fish and then showing the same behaviors was defined as
400 counter-aggression, while the lack of any behaviors upon receiving aggression was defined as
401 received aggression. Partner display was defined as two fish approaching each other holding up
402 their heads and folding their pelvic fins toward their body. This type of behavior is observed
403 when fish form monogamous reproductive pairs [25]. Additionally, rest was defined as a state in
404 which the above behaviors were not exhibited; specifically, pigmentation patterns in this state

were recorded about 1 minute after the other behaviors were observed. Observations of the rest state were conducted randomly five times in eight male and four female adults.

Three types of pigmentation patterns were detected in *S. aequifasciatus* (Fig. 1). Typically, discus fishes have a total of eight vertical bars on their body, including the facial area [41]. However, in this study, individuals did not show vertical bars on their body flank when such bars were absent on their facial area.

Behavioral observation II: effects of facial coloration on aggressive behavior

The objective of these observations was to investigate the impact of facial coloration on the frequency of harassment by dominant individuals toward subordinates. To this end, 10 pairs of mature males (n = 20) were placed within an experimental water tank (162 L, 60 × 45 × 60 cm). In these same-sex experimental pairs, the fish differed in body size by a minimum of 1.5 cm, which ensured the establishment of a size-based hierarchy between them; smaller individuals were designated as subordinates, while larger individuals assumed the role of dominants. The experiment was focused on determining whether the presence of an eye-patch pattern in subordinate individuals served as an emotional signal to mitigate the possibility of continuous aggression (harassment) from dominant individuals, given that eye-patch patterns had been frequently displayed by subordinates in response to aggressive behavior from conspecifics (Fig. 1b).

First of all, upon observing aggressive behavior by dominants against subordinates, the facial coloration of the latter (i.e., absence or presence of the eye-patch pattern) was recorded (hereafter called ‘first aggression’). After the first aggression, whether the dominant individuals continuously attacked the subordinates was also recorded. In this study, an aggressive behavior that occurred within 5 seconds of the first aggression was defined as

429 continuous harassment. If this situation was produced, the facial coloration of subordinates was
430 recorded. In contrast, if dominant individuals did not continuously attack, the facial coloration
431 of subordinates was recorded 5 seconds after the first aggression. If dominant individuals
432 repeated the aggressive behavior three or more times within a single sequence of aggressive
433 behaviors, these were considered as part of the first aggression, and observations continued in
434 the same manner until the continuous harassment ended. During these observations, which
435 lasted for 20 min, a maximum of six consecutive aggressive behaviors against subordinates
436 occurred within a single behavioral sequence. Instances where subordinates did not exhibit the
437 eye-patch pattern upon first aggression by dominants and subsequently maintained their facial
438 coloration without alteration (*A to A*) were observed 97 times. Instances where subordinates did
439 not exhibit the eye-patch pattern upon first aggression but did so after it (*A to P*) were observed
440 69 times. Instances where subordinates exhibited the eye-patch pattern upon first aggression and
441 subsequently altered their facial coloration to a non-eye-patch pattern (*P to A*) were observed 9
442 times. Finally, instances where subordinates exhibited the eye-patch pattern upon first
443 aggression and subsequently maintained their facial coloration without alteration were observed
444 23 times. It should be noted that in 20 cases of aggression, facial coloration could not be
445 discerned; therefore, these data were removed from subsequent analyses
446
447 *Experimental manipulation of social signals*

448 To investigate the potential function of facial coloration as a social signal, body coloration in *S.*
449 *aequifasciatus* was experimentally manipulated using a light treatment known among discus
450 fish enthusiasts that targets the vertical bars, which are believed to serve as social signals.
451 Specifically, this treatment aimed to eliminate the vertical bars by exposing juvenile fish to
452 intense illumination. Initially, mature females and males exhibiting partner displays (Satoh et al.
453 2016) were transferred to separate water tanks (122 L, 60 × 45 × 45 cm or 91 L, 45 × 45 × 45

454 cm). Substrate bricks suitable for spawning were provided in these tanks, and humic acid was
455 periodically added to regulate water quality. Upon spawning, the offspring remained with their
456 parents for 10 days, as early free-swimming fry rely on parental mucus for sustenance (Satoh et
457 al. 2017). Subsequently, 50 fry from the same brood were placed in two smaller tanks. One tank
458 received illumination from a light source positioned approximately 30 cm above, thereby
459 constituting the light-treated group, while the other tank remained under ambient room lighting,
460 serving as the control for wild-type individuals. The juvenile fish were fed *Artemia* spp. nauplii
461 during the experiment. The lighting regime for the light-treated group was synchronized with
462 the room lighting, maintaining a 12-hour light / 12-hour dark cycle. After 3 months, the
463 juveniles were identified and moved to the same water tanks (162 L, 60 × 45 × 60 cm or 182 L,
464 90 × 45 × 45 cm) until they reached sexual maturity, which typically occurs over a period of
465 approximately 1.5–2.0 years. Facial coloration was evaluated in the light-treated individuals in
466 the same manner as in *behavioral observation I*, but none of them exhibited an eye-patch
467 pattern (Supplementary S2).

468 Light-treated and wild-type individuals were used to test whether facial coloration
469 functions analogously to emotional expression. Three types of experimental pairs were created:
470 a group consisting of wild-type individuals only (WW pair, n = 4 male pairs and 4 female pairs),
471 a group of wild-type and light-treated individuals (WL pair, n = 3 male pairs and 3 female
472 pairs), and a group of light-treated individuals only (LL pair, n = 3 male pairs and 4 female
473 pairs). These same-sex experimental pairs (42 individuals in total) were selected to ensure
474 homogeneity in kinship and were allowed to meet each other for the first time. After measuring
475 their body weight in 1 mg, they were placed in a water tank (122 L, 60 × 45 × 45 cm) for 9
476 days. During the experiment, the fish were individually fed with five mosquito larvae per day
477 using tweezers. This was to ensure that the dominant individuals did not monopolize the food.
478 The number of aggressive interactions between pair partners over a period of 20 minutes was

479 counted on days 1, 3, 5, 7, and 9. In this study, aggressive interaction was defined as total
480 aggressive display and direct aggression occurring between pair partners. After 9 days of
481 observations, the body weights of the experimental pairs were measured again. Among the
482 examined groups, individuals exhibiting more aggressive behaviors toward their experimental
483 partners during the experiment were defined as dominant, while those receiving more attacks as
484 submissive. The frequency of aggressive interaction was predicted to be higher in the LL group
485 than in the WW and WL groups if facial coloration has a function in emotional signaling.
486
487 *Tissue clearing and immunohistochemistry*
488 Three bred discus fish *S. aequifasciatus* individuals (22.5–33 mm SL) were used for tissue
489 clearing and immunohistochemistry, primarily following the methods described in Pende et al.
490 (2020)[42]. The fish were deeply anesthetized with benzocaine, and their head and body were
491 sectioned along a plane extending from the base of the pelvic fin to approximately the third to
492 fifth spine of the dorsal fin. After removing the internal organs and creating small holes in the
493 cornea, the heads were fixed in 4% (w/v) paraformaldehyde at 4°C overnight. The specimens
494 were washed four times (30 minutes each) with PBS (pH 7.4) at room temperature and
495 incubated in 0.2 M EDTA (pH 8.0) at 4°C for 48 hours, with the solution refreshed after 24
496 hours. After another four PBS washes at room temperature, they were transferred to pre-chilled
497 acetone and incubated at 4°C for 1 hour. The acetone was then refreshed, and the specimens
498 were incubated overnight at −20°C, followed by another refreshment and incubation overnight
499 at room temperature. After being washed four times with PBS at room temperature, the
500 specimens were treated with 3% (w/w) H_2O_2 in 0.8% (w/v) KOH for about 160 minutes at the
501 same temperature under intense light exposure. Subsequently, they were washed with 1% (v/v)
502 Triton X-100 in PBS (PBST) for two nights at 4°C and placed under vacuum for 60 minutes to
503 remove gas bubbles. They were then incubated in Solution-1.1 (pH 11.5) for two nights at 37°C,
504 with the solution being refreshed after the first night. This solution consisted of 10% (v/v)

505 THEED (Sigma-Aldrich, 87600-100 ml), 5% (v/v) Triton X-100, and 5% (w/v) urea in dH_2O,
506 and its pH was adjusted to 11.5 using 30% (w/w) ammonia solution. After five PBST washes
507 (10 minutes each), the specimens were treated with 1% (w/v) bovine serum albumin (BSA;
508 Proliant Biologicals, PRL68700-25G) in PBST overnight at 4°C. Then, they were incubated in
509 1:1,000 primary antibody (rabbit anti-TH; Merck Millipore, AB152) in PBST with 0.5% BSA
510 for 4 days at 4°C, followed by [*add number*] PBST washes for 2 days at 4°C. Next, the
511 specimens were incubated in 1:200 biotinylated goat anti-rabbit IgG secondary antibodies
512 (Vector Laboratories, BA-1000) in PBST with 0.5% BSA for 4 days at 4°C, washed in PBST for
513 2 days at 4°C, and incubated in a mixture of 1:100 avidin and 1:100 biotinylated horseradish
514 peroxidase (Vector Laboratories, PK-6100) in PBST for 4 days at 4°C, followed by PBST
515 washes for 2 days at 4°C. The specimens were stained in a reaction solution containing 0.05%
516 (w/v) 3,3'-diaminobenzidine, 0.01% H_2O_2, 0.04% (w/v) nickel ammonium sulfate, 0.5% (v/v)
517 Triton X-100, and 0.1 M phosphate buffer (pH 7.4) in ultra-pure water, followed by [*add*
518 *number*] PBST washes for 1 day at room temperature. For refractive index matching, the three
519 specimens were incubated in CUBIC-R+(N) (45% [w/w] antipyrine, 30% [w/w] nicotinamide,
520 0.5% [v/v] N-butyldiethanolamine in ultra-pure water) (Matsumoto et al. 2019) for 3 days at
521 room temperature. The eye-patch regions were then observed and photographed under a
522 microscope fitted with a digital camera (Olympus, SZX10 with DP25).
523
524 *Ex vivo administration of NA and ATP to scale melanophores*
525 Three bred discus fish *S. aequifasciatus* individuals (21–24 mm SL) were anesthetized with
526 benzocaine, and a total of 20 scales (ATP exposure group, n = 5; control for ATP exposure
527 group, n = 5; NA exposure group, n = 5; control for NA exposure group, n = 5) were collected
528 from the eye-patch region and kept in ice-cold PBS for a short period until the experiment was
529 started. The scales were placed on a concave slide glass filled with PBS, secured by pinching
530 the edges with a polypropylene sheet, and observed under a microscope (Olympus, BX53). No

531 melanophores were present in the pinched areas. It was difficult to completely standardize the
532 physiological state of each individual at the time of scale collection and the neural innervation
533 status across all scales. Therefore, the ATP exposure group was pre-treated with NA, and the NA
534 exposure group was pre-treated with ATP, and changes in melanosome movement due to
535 neurotransmitter exposure administration were specifically quantified. After removing the PBS
536 from the concave slide, 0.1 mM ATP in PBS was added to the NA exposure group, and 0.1 μM
537 NA in PBS was added to the ATP exposure group, followed by incubation at room temperature
538 for 2 minutes. Subsequently, the solutions were removed, and the scales were washed by adding
539 and quickly removing PBS. A total of 1 μM NA in PBS or PBS was added to the NA exposure
540 group, and a total of 10 mM ATP in PBS or PBS was added to the ATP exposure group.
541 Considering the moment each solution was applied as 0 seconds, photographs were taken using
542 a microscope fitted with a camera (Olympus, BX53 with DP74) at 1 second and every 10
543 seconds after that for 120 seconds. All images were adjusted for white balance using Adobe
544 Photoshop (version 25.9.1) and analyzed using ImageJ (version 2.14.0.). To exclude
545 xanthophores, the images were split into the three primary colors, and each monochrome one
546 was used in the analysis. Subsequently, one well-captured melanophore (e.g., focus alignment)
547 was randomly selected per scale, and its area was set as the ROI. Then, the area showing a
548 grayscale above the identical threshold for all images within the ROI was calculated. The rate of
549 variation in melanosome dispersion (%) was calculated using the following formula:

550 $$Rate\ of\ change\ in\ melanosome\ dispersion$$

551 $$= \frac{Final\ extent\ of\ melanosome\ dispersion}{Initial\ extent\ of\ melanosome\ dispersion} \times 100\ (\%)$$

552

553 *Statistical analysis*

554 All statistical analyses were performed in R (v. 4.3.3) [77] using LMMs or GLMMs in the lme4
555 package [78].

To evaluate whether facial coloration in subordinate individuals mitigated harassment by dominant individuals, the probability of continued harassment was analyzed as a binary variable within a binomial GLMM, with experimental pair ID incorporated as a random effect. The variation in facial coloration, specifically whether the facial pattern transitioned before or after the initial aggression by dominants (A to A, A to P, P to A, or P to P), was included as an explanatory variable. Given that changes in facial coloration were observed to significantly affect the likelihood of continuous harassment, post-hoc testing was conducted using separate binomial GLMMs, again treating experimental pair ID as a random effect. For these post-hoc GLMMs, the significance level (α) was adjusted via Bonferroni correction to $\alpha = 0.0083$.

With regard to the experimental manipulation of facial coloration, a negative binomial GLMM was employed to analyze the data, with the experimental pair ID included as a random effect. This model examined the two-way interaction between the experimental groups (WW, WL, and LL pairs) and observation days (1, 3, 5, 7, and 9) as explanatory variables. Furthermore, the reduction in body weight during the experiment was analyzed using an LMM, again incorporating experimental pair ID as a random effect. The response variable was the percentage reduction in body weight (calculated as post-experiment body weight divided by pre-experiment body weight). The explanatory variables included the experimental group (WW, WL, and LL pairs), the sex of focal individuals, their status (light-treated or wild-type), and their social rank (dominant or subordinate).

Finally, the rate of variation in melanosome dispersion was examined upon exposure to ATP and NA, which act as sympathetic neurotransmitters. Prior to analysis, the black area in the selected melanophore (measured in pixels) was log-transformed (log10) to achieve normalization. An LMM was employed in this analysis, with individual ID included as a random effect. The two-way interaction between time and treatment (either ATP or PBS control) was modeled [*using?*] as an explanatory variable. An analogous analysis was conducted for the

NA experiment. It should be noted that the results of these analyses are presented in Fig. 5 as the percentage change in melanosome dispersion relative to the baseline measurements at the start of the experiment for visualization.

References

1. Rhodes, G., Simmons, L., Barrett, L. & Dunbar, R. Oxford Handbook of Evolutionary Psychology (2007).

2. McKone, E., Kanwisher, N. & Duchaine, B. C. Can generic expertise explain special processing for faces ? Trends Cogn. Sci. 11, 8–15 (2007)

3. Schmidt, K. L. & Cohn, J. F. Human facial expressions as adaptations: evolutionary questions in facial expression research. Am. J. Phys. Anthropol. 116, 3–24 (2001).

4. Kobayashi, H. & Kohshima, S. Unique morphology of the human eye. Nature 387, 767–768 (1997).

5. Tomasello, M., Hare, B., Lehmann, H. & Call, J. Reliance on head versus eyes in the gaze following of great apes and human infants: the cooperative eye hypothesis. J. Hum. Evol. 52, 314–320 (2007).

6. Rinn, W. E. The neuropsychology of facial expression: a review of the neurological and psychological mechanisms for producing facial expressions. Psychol. Bull. 95, 52–77 (1984).

7. Cattaneo, L. & Pavesi, G. The facial motor system. Neurosci. Biobehav. Rev. 38, 135–159 (2014).

8. Müri, R. M. Cortical control of facial expression. J. Comp. Neurol. 524, 1578–1585 (2016).

9. Allison, T., Puce, A. & McCarthy, G. Social perception from visual cues: role of the STS region. Trends Cogn. Sci. 4, 267–278 (2000).

10. Engell, A. D. & Haxby, J. V. Facial expression and gaze-direction in human superior temporal sulcus. Neuropsychologia 45, 3234–3241 (2007).

11. Xu, P., Peng, S., Luo, Y. J. & Gong, G. Facial expression recognition: a meta-analytic review of theoretical models and neuroimaging evidence. Neurosci. Biobehav. Rev. 127, 820–836 (2021).

12. Hadders-Algra, M. Human face and gaze perception is highly context specific and involves bottom-up and top-down neural processing. Neurosci. Biobehav. Rev. 132, 304–323 (2022).

13. Ekman, P. & Friesen, W. V. Constants across cultures in the face and emotion. J. Pers. Soc. Psychol. 17, 124–129 (1971).

14. Boakes, R. From Darwin to Behaviourism: Psychology and the Minds of Animals (Cambridge Univ. Press, New York, 1984).

15. Ekman, P. Darwin and Facial Expression: A Century of Research (2006).

16. Darwin, C. The Expression of the Emotions in Man and Animals (1872).

17. Parr, L. A., Waller, B. M. & Heintz, M. Facial expression categorization by chimpanzees using standardized stimuli. Emotion 8, 216–231 (2008).

18. Vick, S. J., Waller, B. M., Parr, L. A., Smith Pasqualini, M. C. & Bard, K. A. A cross-species comparison of facial morphology and movement in humans and chimpanzees using the facial action coding system (FACS). J. Nonverbal Behav. 31, 1–20 (2007).

19. Leopold, D. A. & Rhodes, G. A comparative view of face perception. J. Comp. Psychol. 124, 233–251 (2010).

20. Langford, D. J. et al. Coding of facial expressions of pain in the laboratory mouse. Nat. Methods 7, 447–449 (2010).

21. Bertin, A. et al. Captive Blue-and-yellow macaws (*Ara ararauna*) show facial indicators of positive affect when reunited with their caregiver. Behav. Processes 206, 104833 (2023).

22. Tate, A. J., Fischer, H., Leigh, A. E. & Kendrick, K. M. Behavioural and neurophysiological evidence for face identity and face emotion processing in animals. Philos. Trans. R. Soc. Lond. B Biol. Sci. 361, 2155–2172 (2006).

23. Demaria, C. & Thierry, B. Responses to animal stimulus photographs in stumptailed macaques (*Macaca arctoides*). Primates 29, 237–244 (1988).

24. Guo, K., Robertson, R. G., Mahmoodi, S., Tadmor, Y. & Young, M. P. How do monkeys view faces?–A study of eye movements. Exp. Brain Res. 150, 363–374 (2003)

25. Satoh, S., Tanaka, H. & Kohda, M. Facial recognition in a discus fish (Cichlidae): experimental approach using digital models. PLOS ONE 11, e0154543 (2016).

26. Siebeck, U. E., Parker, A. N., Sprenger, D., Mäthger, L. M. & Wallis, G. A species of reef fish that uses ultraviolet patterns for covert face recognition. Curr. Biol. 20, 407–410 (2010).

27. Kohda, M. et al. Facial recognition in a group-living cichlid fish. PLOS ONE 10, e0142552 (2015).

28. Wang, M. Y. & Takeuchi, H. Individual recognition and the 'face inversion effect' in medaka fish (Oryzias latipes). Elife, 6, e24728 (2017)

29. Saeki, T., Sogawa, S., Hotta, T. & Kohda, M. Territorial fish distinguish familiar neighbours individually. Behaviour 155 , 279–293 (2018).

30. Hotta, T., Kawasaka, K., Satoh, S. & Kohda, M. Fish focus primarily on the faces of other fish. Sci. Rep. 9, 8377 (2019).

31. Sogawa, S. et al. Male guppies recognize familiar conspecific males by their face. Zool. Sci. 40, 168–174 (2023).

32. Kawasaka, K., Hotta, T. & Kohda, M. Does a cichlid fish process face holistically? Evidence of the face inversion effect. Anim. Cogn. 22, 153–162 (2019).

33. Kohda, M. et al. Cleaner fish recognize self in a mirror via self-face recognition like humans. Proc. Natl Acad. Sci. U. S. A. 120, e2208420120 (2023).

34. Kano, F. & Tomonaga, M. How chimpanzees look at pictures: a comparative eye-tracking study. Proc. Biol. Sci. 276, 1949–1955 (2009).

35. Farias, I. P. & Hrbek, T. Patterns of diversification in the discus fishes (*Symphysodon* spp. Cichlidae) of the Amazon Basin. Mol. Phylogenet. Evol. 49, 32–43 (2008).

36. Crampton, W. G. Ecology and life history of an Amazon floodplain cichlid: the discus fish Symphysodon (Perciformes: Cichlidae). Neotrop. Ichthyol. 6, 599–612 (2008).

37. Satoh, S., Tanoue, H., & Mohri, M. Costs and benefits of biparental mucus provisioning in discus fish (*Symphysodon aequifasciatus*). Ichthyol Res, 65, 510-514 (2018)

38. Satoh, S., & Sowersby, W. Mucus provisioning behavior in teleost fishes: a novel model system for the evolution of secretory provisioning in vertebrates. Ichthyol Res, 68, 1-10 (2021)

39. Satoh, S., Tanoue, H., Ruitton, S., Mohri, M., & Komatsu, T. Morphological and behavioral ontogeny in larval and early juvenile discus fish *Symphysodon aequifasciatus*. Ichthyol Res, 64, 37-44 (2017)

40. Muske, L. E. & Fernald, R. D. Control of a teleost social signal. II. Anatomical and physiological specializations of chromatophores. J. Comp. Physiol. A 160, 99–107 (1987).

41. Bleher, H. Bleher's Discus (Aquapress, Germany, 2006).

42. Pende, M. et al. A versatile depigmentation, clearing, and labeling method for exploring nervous system diversity. Sci. Adv. 6, eaba0365 (2020).

43. Balzarini, V., Taborsky, M., Villa, F., & Frommen, J. G. Computer animations of color markings reveal the function of visual threat signals in *Neolamprologus pulcher.* Curr Zool, 63(, 45-54 (2017)

44. Muske, L. E. & Fernald, R. D. Control of a teleost social signal. I. Neural basis for differential expression of a color pattern. J. Comp. Physiol. A 160, 89–97 (1987).

45. Rodrigues, R. R., Carvalho, L. N., Zuanon, J., & Del-Claro, K. Color changing and behavioral context in the Amazonian Dwarf Cichlid *Apistogramma hippolytae* (Perciformes). Neot Ichthyo, 7, 641-646 (2009)

46. Seikai, T. Influences of fluorescent light irradiation, ocular side pigmentation, and source of fishes on the blind side pigmentation in the young Japanese flounder, Paralichthys olivaceus. Suisanzoshoku 39, 173–180 (1991).

47. Andersson, M. Sexual Selection (Princeton Univ. Press, 1994).

48. Krause, J. & Ruxton, G. D. Living in Groups (Oxford Univ. Press, Oxford, 2002).

49. Fujii, R. & Oshima, N. Control of chromatophore movements in teleost fishes. Zool. Sci. 3, 13–47 (1986).

50. Fujii, R. The regulation of motile activity in fish chromatophores. Pigment Cell Res. 13, 300–319 (2000).

51. Sugimoto, M. Morphological color changes in fish: regulation of pigment cell density and morphology. Microsc. Res. Tech. 58, 496–503 (2002).

52. von Frisch, K. Beiträge zur physiologie der pigmentzellen in der fischhaut. Pflüger's Arch. 138, 319–387 (1911).

53. Fujii, R. & Novales, R. R. The nervous mechanism controlling pigment aggregation in *Fundulus melanophores*. Comp. Biochem. Physiol 29, 109–124 (1969).

54. Funakoshi, K. & Nakano, M. The sympathetic nervous system of anamniotes. Brain Behav. Evol. 69, 105–113 (2007).

55. Nilsson Sköld, H., Aspengren, S. & Wallin, M. Rapid color change in fish and amphibians – function, regulation, and emerging applications. Pigment Cell Melanoma Res. 26, 29–38 (2013).

56. Konno, A., Matsumoto, N., Tomono, Y. & Okazaki, S. Pathological application of carbocyanine dye-based multicolour imaging of vasculature and associated structures. Sci. Rep. 10, 12613 (2020).

57. Ueda, H. R. et al. Tissue clearing and its applications in neuroscience. Nat. Rev. Neurosci. 21, 61–79 (2020).

58. Matsumoto, K. et al. Advanced CUBIC tissue clearing for whole-organ cell profiling. Nat. Protoc. 14, 3506–3537 (2019).

59. Yew, D. T., Webb, S. E. & Lam, E. T. Neurotransmitters and peptides in the developing human facial nucleus. Neurosci. Lett. 206, 65–68 (1996)

60. Leong, C. Y. The quantitative effect of releasers on the attack readiness of the fish *Haplochromis burtoni* (Cichlidae, Pisces). Z Vergl Physiol. 65, 29–50 (1969).

61. Heiligenberg, W., Kramer, U. & Schulz, V. The angular orientation of the black eye-bar in *Haplochromis burtoni* (Cichlidae, Pisces) and its relevance to aggressivity. Z Vergl Physiol. 76, 168–176 (1972).

62. Schmidt, K. L. & Cohn, J. F. Human facial expressions as adaptations: evolutionary questions in facial expression research. Am. J. Phys. Anthropol. 116, 3–24 (2001).

63. Straulino, E., Scarpazza, C. & Sartori, L. What is missing in the study of emotion expression? Front. Psychol. 14, 1158136 (2023).

64. Bshary, R. Biting cleaner fish use altruism to deceive image–scoring client reef fish. Proc. Biol. Sci. 269, 2087–2093 (2002).

65. Fernald, R. D. Decision-making in fish: genetics and social behaviour. In Dreher, J. C. & Tremblay, L. (eds.), Decision Neuroscience: An Integrative Perspective 351–359 (Academic Press, San Diego, 2017).

66. de Melo, C. M. & Gratch, J. Expression of emotions using wrinkles, blushing, sweating and tears. In: Intelligent Virtual Agents: International Conference, IVA 2009 Amsterdam, The Netherlands, September 14-16 (Springer Berlin Heidelberg, 2009).

67. Lee, D. H., Susskind, J. M. & Anderson, A. K. Social transmission of the sensory benefits of eye widening in fear expressions. Psychol. Sci. 24, 957–965 (2013).

68. Ruxton, G. D., Allen, W. L., Sherratt, T. N. & Speed, M. P. Avoiding Attack: The Evolutionary Ecology of Crypsis, Aposematism, and Mimicry (Oxford Univ. Press, 2019).

69. Wendelaar Bonga, S. E. The stress response in fish. Physiol. Rev. 77, 591–625 (1997).

70. Barton, B. A. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr. Comp. Biol. 42, 517–525 (2002).

71. Madaro, A. et al. How fish cope with stress?. In: Kristiansen, T., Fernö, A., Pavlidis, M. van de Vis. H. (eds.), The Welfare if Fish (Springer Nature, Switzerland, 2020).

72. Fox, H. E., White, S. A., Kao, M. H. & Fernald, R. D. Stress and dominance in a social fish. J. Neurosci. 17, 6463–6469 (1997).

73. Sloman, K. A., Metcalfe, N. B., Taylor, A. C. & Gilmour, K. M. Plasma cortisol concentrations before and after social stress in rainbow trout and brown trout. Physiol. Biochem. Zool. 74, 383–389 (2001).

74. Schjolden, J. & Winberg, S. Genetically determined variation in stress responsiveness in rainbow trout: behavior and neurobiology. Brain Behav. Evol. 70, 227–238 (2007).

75. Denil, N. A., Yamashita, E., Kirino, M. & Kiyohara, S. Recurrent facial taste neurons of sea catfish *Plotosus japonicus*: morphology and organization in the ganglion. J. Fish Biol. 82, 1773–1788 (2013).

76. Albohn, D. N. & Adams Jr, R. B. The social face hypothesis. Affect. Sci. 3, 539–545 (2022).

77. R Core Team. R: a language and environment for statistical computing. Version 4.3.3. https://www.R-project.org/ (2023).

78. Bates, D., Maechler, M. & Bolker, B. lme4: linear mixed effects models using S4 classes. http://CRAN.R-project.org/package=lme4 (2011).

79. Satoh, S., et al. Data from “Facial coloration and behaviors of discus fish”. Figshare (___)

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Author contribution

S.S., K.F., A.K., S.T., M.K., and N.K. designed the study. S.S., K.F., S.M., T.B., and K.K. collected the data. S.S. and K.F. conducted the analyses. S.S. and K.F. wrote the manuscript with input from all the authors.

Competing interests

The authors declare no competing interests.

Ethics statement

All the experimental protocols were approved by the Animal Care and Use Committees at Osaka City University and Kitazato University and adhered to the ASAB/ABS guidelines for the treatment of animals in behavioral research.

Data availability

The data that supports the findings of this study are available in the figshare repository XXX [79].

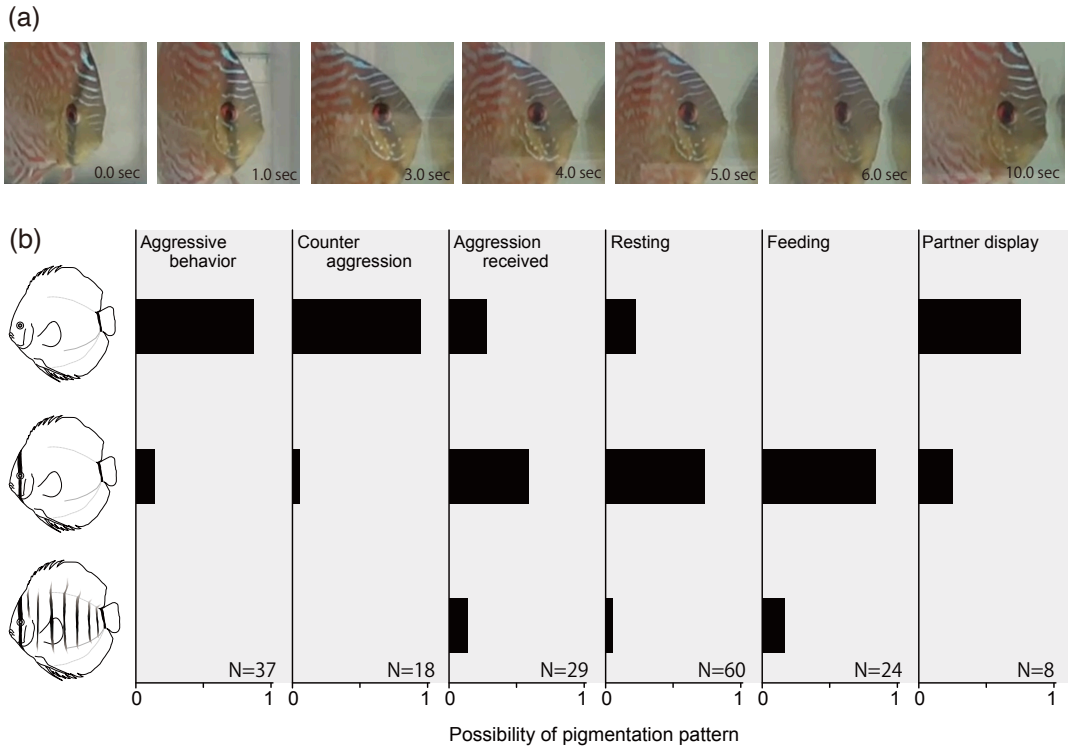


Figure 1 Facial coloration in discus fish *Symphysodon aequifasciatus*. Typical chronological changes of eye-patch pattern (a) and coloration associated with social behaviors (b). The bars represent the possibility of coloration patterns emerging when individuals engage in specific social behaviors. Three identifiable coloration patterns were detected: 1) absence of any vertical black bar composed of melanophores; 2) presence of only one bar consisting of melanophores, eye-patch pattern, and 3) presence of multiple bars throughout the body.

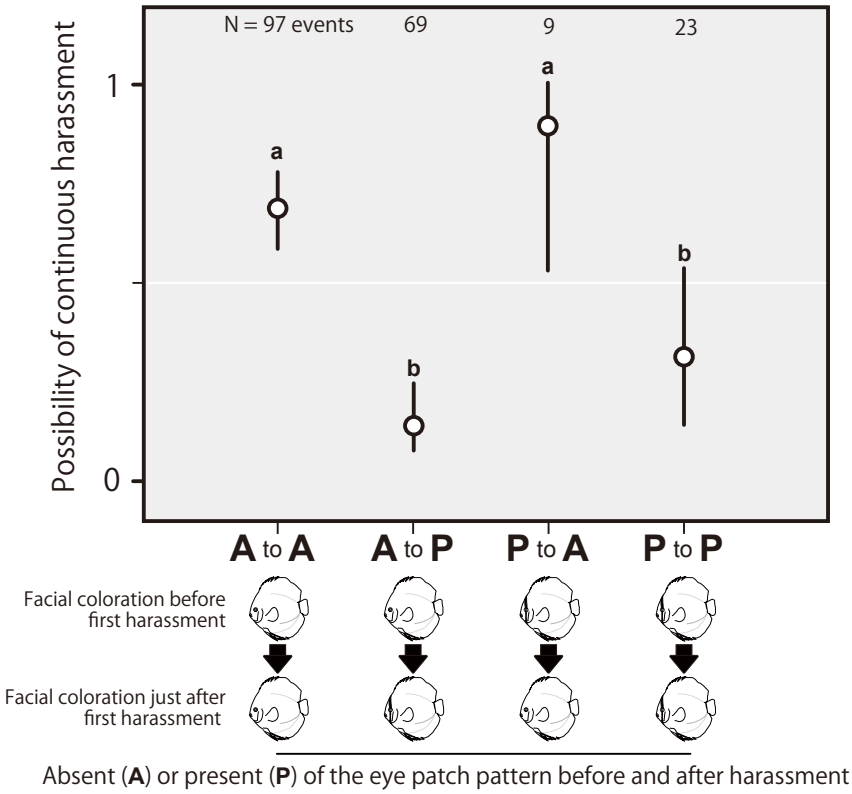


Figure 2 Effects of facial coloration in *S. aequifasciatus* subordinates on the continuous aggressive harassment by dominant individuals. The data are presented as mean values along with their corresponding 95% confidence intervals, and the sample sizes are indicated above the bars. Significant differences were determined using post-hoc binomial GLMMs, with p-values adjusted using Bonferroni correction (α level = 0.0083). The possibility of experiencing continuous harassment was notably higher in *A to A* than in *A to P* (df = 1, χ^2 = 54.186, p < 0.001) and *P to P* (df = 1, χ^2 = 10.05, p = 0.002), but similar to that of *P to A* (df = 1, χ^2 = 2.093, p = 0.148). Conversely, the probability of continuous harassment was significantly lower in *A to P* than in *P to A* (df = 1, χ^2 = 22.076, p < 0.001), but similar to that of *P to P* (df = 1, χ^2 = 3.3123, p = 0.0688). Additionally, there was a significant difference in the possibility of continuous harassment between *P to A* and *P to P* (df = 1, χ^2 = 9.690, p = 0.002).

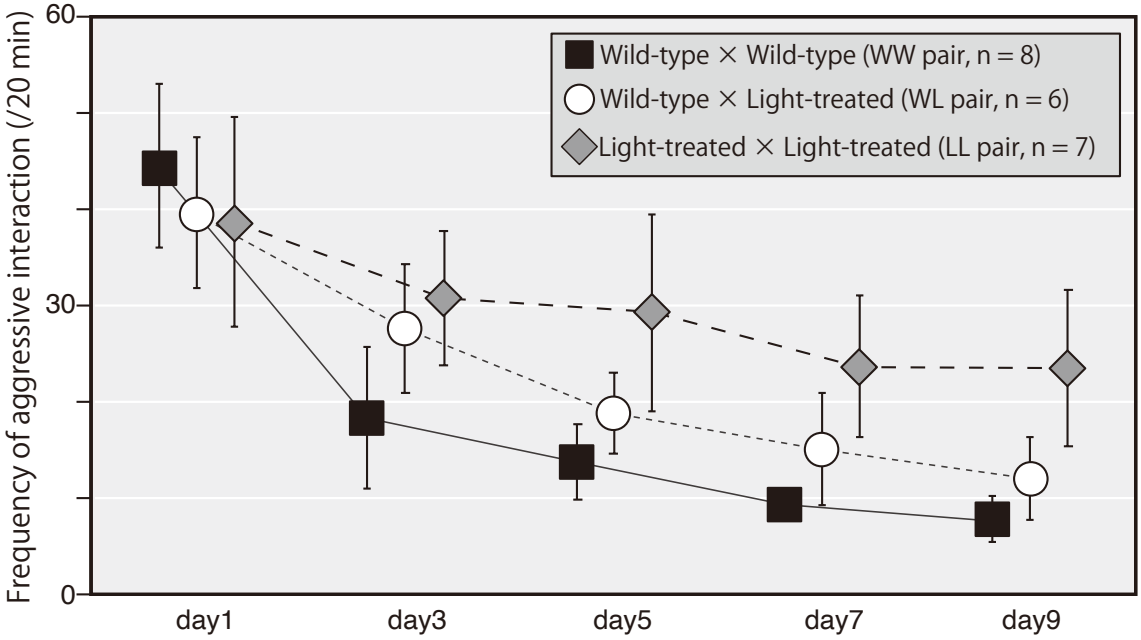


Figure 3 Effects of the experimental manipulation of melanophores on aggressive social interaction in *S. aequifasciatus*. The figure shows the relationship between the frequency of aggressive interaction within experimental groups and days since the start of the experiment. The experimental groups, consisting of wild-type × wild-type individuals (WW), wild-type × light-treated individuals (WL), and light-treated × light-treated individuals (LL), are indicated by black, white, and gray bars, respectively. A significant interaction was detected between the type of experimental group and the duration of the experiment (negative binomial GLMM, df = 2, $\chi^2 = 67.896$, $p < 0.001$). Data are presented as mean values ± standard deviation (SD).

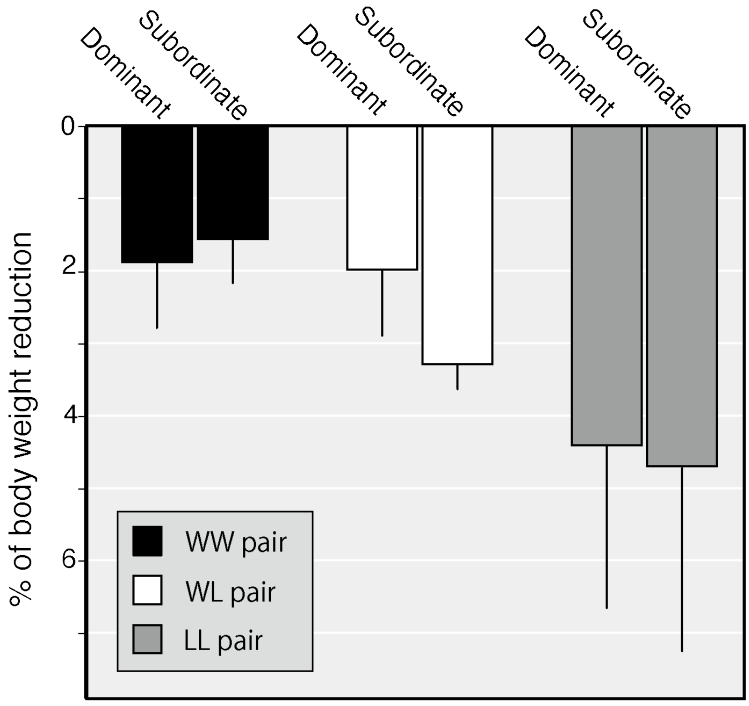


Figure 4 Reduction in body weight during the 9-day experiment in dominant and subordinate *S. aequifasciatus*. The experimental groups, consisting of wild-type × wild-type individuals (WW), wild-type × light-treated individuals (WL), and light-treated × light-treated individuals (LL), are indicated by black, white, and gray bars, respectively. Significant differences were detected in the percentage of reduction in body weight between experimental groups (LMM, df = 2, F = 4.398, p = 0.025), while marginal differences were observed in dominance rank (df = 1, F = 3.955, p = 0.061). Data are presented as mean values ± standard deviation (SD).

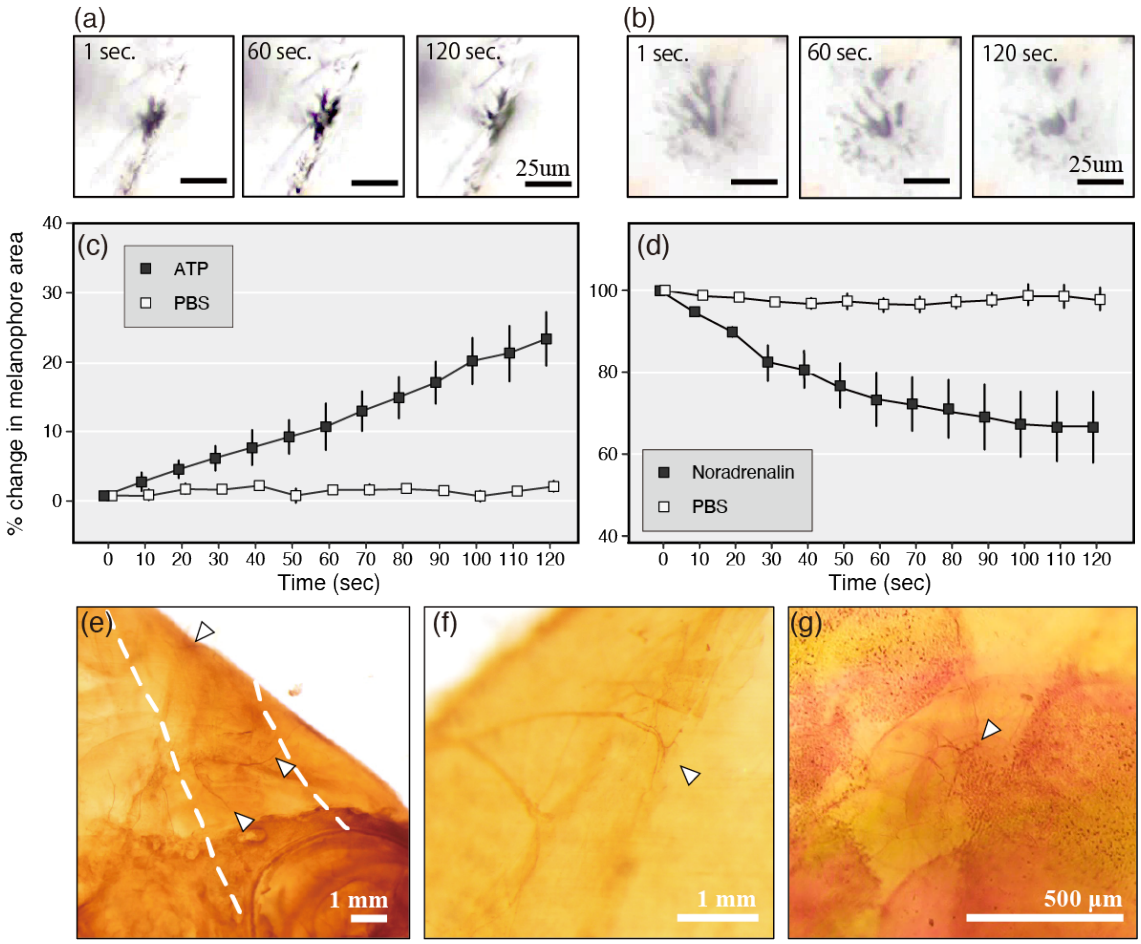
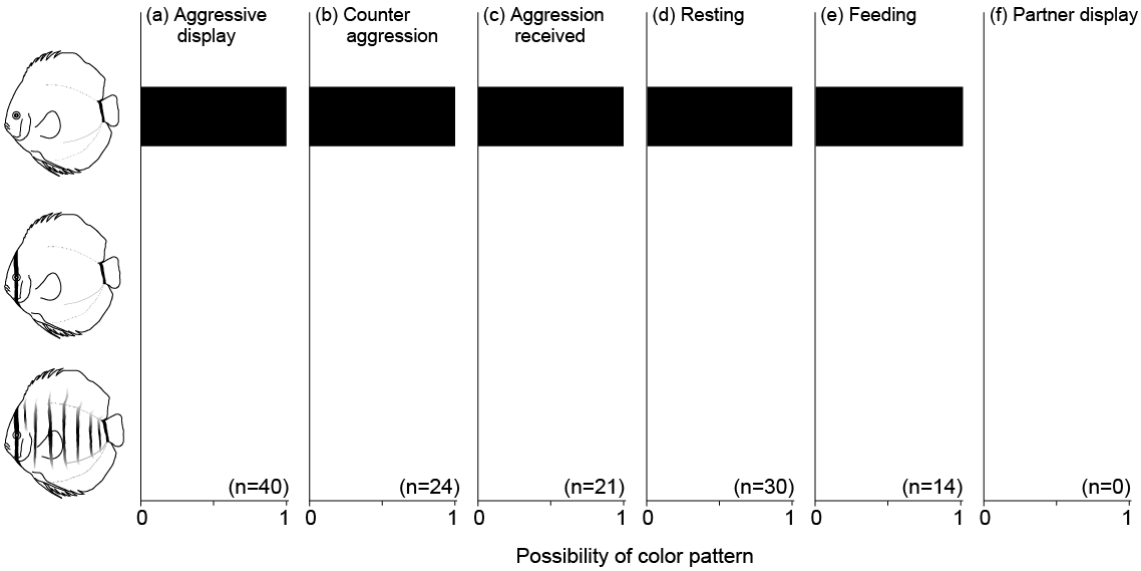


Figure 5 Effect of the noradrenaline (NA) and adenosine triphosphate (ATP) neurotransmitters used by the sympathetic postganglionic neurons on melanophores constituting the eye patch and tyrosine hydroxylase-immunoreactive projection to the eye-patch region. The peripheral nerves regulating the melanophores constituting the eye patch were examined. **a–d** Temporal variations in the extent of melanosome dispersion upon the exposure of melanophores constituting the eye patch to NA and ATP . The rate of variation in melanosome dispersion upon exposure to ATP (a, c) and NA (b, d) was significantly different from that observed in the PBS control (two-way interaction between time and treatment, both $p < 0.001$). Data are presented as mean values ± standard error. **e–g** The tissues were cleared and immunostained using an antibody against tyrosine hydroxylase (TH), revealing that TH-immunoreactive (TH-ir) projections extended to the eye-patch region (white arrowheads). The

white dotted lines indicate the eye-patch region (**e**). The observations strongly suggested that TH-ir fibers terminate at the scales possessing melanophores forming the eye patch: (**b**) specimen whose scales had been removed to improve the visibility of the TH-ir fibers and **(e)** specimen with intact scales.

Supplementary

Supplemental movie S1 Typical example of facial coloration transition in the discus fish *Symphysodon aequifasciatus*. To facilitate the viewing of changes in facial coloration patterns, we presented a model of the reproductive partner on the monitor and observed its behavior based on the method described in Satoh et al. (2016) [25].



Supplemental Figure S2 Coloration associated with social behaviors in light-treated *S. aequifasciatus*. The bars represent the possibility of coloration patterns emerging when individuals engage in specific social behaviors. Three identifiable coloration patterns were detected: 1) absence of any vertical black bar composed of melanophores; 2) presence of only one bar consisting of melanophores, eye-patch pattern, and 3) presence of multiple bars throughout the body. These observations were made in the same manner as in *behavioral observation I* (see Methods).