



Male mating tactics and secondary sexual traits: insights from the Mediterranean killifish, *Aphanius fasciatus*

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ABSTRACT

Context. Sexual selection results from male competition and female choice linked by complex interactions that drive the population's life history, often leading to the development of specific secondary sexual traits with an increased reproductive success. **Aims.** To investigate the male mating tactics and the role of a secondary sexual trait ('black band' on the caudal fin) along the male ontogenetic development in the Mediterranean killifish, *Aphanius fasciatus* (Valenciennes, 1821). **Methods.** Male–male competition was investigated through a combination of an extensive field collection and behavioural analysis under controlled conditions, comparing four groups differing in the presence or absence of the black band and body size. **Key results.** Banded males courted more and tended to monopolise the female, but the overall spawning performance did not differ significantly among the analysed groups. **Conclusion.** (1) The black band on the caudal fin depends on ontogeny; (2) all mature males express the entire courtship and competitive repertory; (3) sexual maturity occurs in fish of at least 20 mm; (4) large size and presence of the black band seem to contribute to male dominance. **Implications.** This study has highlighted the role of *Aphanius fasciatus* as an excellent model for studying polygynandrous mating systems, on the basis of intense male–male competition.

Keywords: *Aphanius fasciatus*, Cyprinodontidae, fish behaviour, killifish, life history, male–male competition, mating behaviour, secondary sexual traits, sexual selection.

Introduction

A key aspect to understand the population structure and dynamics of a species is its mating system. Sexual selection acts on the life history of a population by favouring the success of specific genes and phenotypic traits. In the tropical fish *Poecilia reticulata*, for example, the colour and brightness of males are the results of sexual traits evolved from the mating choice of females who prefer bright colours (Kodric-Brown 1985, 1989; Houde 1987; Kodric-Brown and Nicoletto 1993; Grether 2000). In another fish species (*Cyprinodon pecosensis* Echelle & Echelle, 1978), the size and colour of individuals are positively correlated with the intensity and quality of courtship, allowing females to choose more conspicuous traits as indicators of good condition, good social status, and the ability to defend territory (Kodric-Brown 1990). Hence, sexual selection actively acts on gene transmission and thus drives the life history of a population. However, not only female choice is involved in this process. Indeed, as stated by Darwin (1872), sexual selection is the result of male competition and female choice linked by complex interactions that could influence the evolution of sexual characteristics in males. Male competitive behaviour plays a key role in sexual selection.

In fish, it is possible to observe various tactics to maximise the probability of fertilising eggs, such as (1) monopolising resources by aggressive behaviour, (2) fertilising some of the eggs by deceiving the dominant male through sneak–chase behaviour (sneaker strategy), (3) reaching the female or the eggs first, and (4) paying the cost of cooperation with other individuals to guarantee a share of fertilisable eggs (Fuller 2001; Taborsky 2001; McGhee *et al.* 2007). Scientific literature documents many examples where the males with the

ability to dominate over others or with the phenotypic traits associated with dominant status have more opportunities to monopolise females. McGhee et al. (2007) showed that males with more motivation and aggressive attitudes in courtship had better access to females and egg fertilisation. Doutrelant and McGregor (2000) showed that *Betta splendens* females (commonly known as Siamese fighting fish) who witnessed aggressive interactions between two males tended to interact with the winning male and not the defeated one.

Killifishes represent an excellent model for understanding the interactions between male competition and female choice, as large aggregations of individuals often characterise the reproductive system in a specific reproductive area where males attempt to monopolise the female or fertilise the eggs through courtship, territorial defence, aggressive behaviour and sneaking (Kodric-Brown 1986; Fuller 2001; Leiser and Itzkowitz 2002, 2004; McGhee et al. 2007; Malavasi et al. 2010; Cavraro et al. 2013). Studying these dynamics in controlled conditions and reproducing a natural-like situation can provide information on the mating system (Fuller 2001), fish behaviour (Kodric-Brown 1986) and the role of secondary sexual traits (Pizzolon et al. 2008; Johnson and Fuller 2015). In such a system characterised by polygyny, the primary male tends to monopolise the females, thus forcing the secondary males to adopt alternative tactics such as the sneaking strategy (Leiser and Itzkowitz 2004). In the genus *Fundulus*, eggs spawn and fertilisation occurs in shallow water, with males competing for physical proximity to the females, often leading to multiple spawning (Paterson and Whitfield 2000). Lott (1979) showed that in the sheepshead pupfish (*Cyprinodon variegatus*), the primary male's reproductive success decreases when the number of secondary males increases. In this case, the dominant male reduces his status and invests more energy in aggressive behaviour and territoriality in a smaller breeding ground. These observations suggest that in killifish there is a direct investment in courtship and aggressive behaviours to primarily access or monopolise the females (Cavraro et al. 2013).

The Mediterranean killifish *Aphanius fasciatus* (Valenciennes, 1981), commonly named nono, can be considered the ecological equivalent of the North American *Fundulus* spp. It is adapted to European salt marshes and has therefore been extensively studied in terms of reproductive behaviours. It shows a breeding system characterised by high levels of male–male competition and an intense regime of sexual selection where males aggregate in high numbers in vegetated areas and compete with each other, trying to monopolise the females. This system leads to a repertoire of courtship behaviours that often end in multiple spawning, with peripheral males trying to fertilise eggs by sneaking (Grech and Schembri 1993; Malavasi et al. 2010; Cavraro et al. 2013). *A. fasciatus*

ethogram of male reproductive behaviours is well described (Malavasi et al. 2010; Cavraro et al. 2013); still, there is a lack of knowledge concerning the role and distribution across its natural populations of a specific sexual secondary trait, namely a black vertical band on the distal portion of the yellowish-orange caudal fin (Fig. 1). Dark vertical bands in *A. fasciatus* males are most likely to be based on melanin (Cavraro et al. 2021) and could be involved in the reproductive behaviour. Previous observations (Altavilla 2022) have shown that this band is visible during the reproductive season only on some males of the male breeding population. Indeed, in the bluefin killifish, *Lucania goodei*, melanin pigmentation was positively correlated with dominance and spawning success (Johnson and Fuller 2015). The size of sexual maturity, when the juveniles *A. fasciatus* participates in the breeding system, and how the mating repertoire differs between big and motivated individuals and young smaller ones, are also unknown. Acquiring this information might help in understanding the mating system and other related aspects for this species, such as the life-history strategy and the predictors of male reproductive success. In this paper, we investigated (a) the male–male competition and the male mating tactics, in all the mature males of the population along the male ontogenetic gradient, and (b) the role of a male trait in the mating system of the Mediterranean killifish, *A. fasciatus* (presence or absence of a black vertical band in the yellowish-orange caudal fin). The present study combined the following two approaches: (1) large field collection of a male population across the breeding season, looking at the distribution and occurrence of the investigated sexual trait, and (2) behavioural analysis under aquarium conditions simulating the competition between two males for the access to a receptive female. If the caudal dark band is indeed a secondary sexual character involved in the reproductive behaviour of *A. fasciatus*, then we hypothesised that it could be a male-dominance signal, being therefore related to a greater mating success.

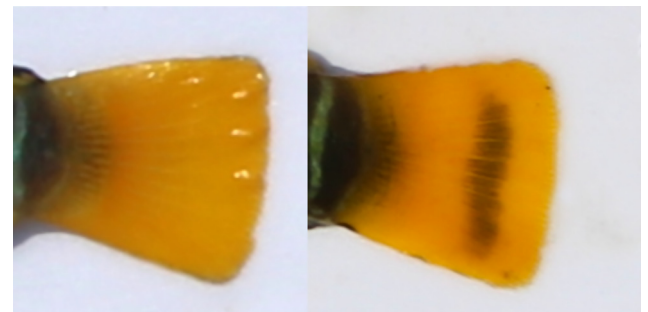


Fig. 1. Caudal fin of *Aphanius fasciatus* without (left) and with (right) the phenotypic trait black vertical band.

Materials and methods

The study species

Aphanius fasciatus (Actinopterygii: Cyprinodontiformes) is a small killifish with a short life cycle that prefers little creeks and pools characterised by shallow water with low predation pressure. Individuals present sexual dimorphism (Fig. 2) and are small in size, with the male being smaller than the female that can reach up to 9 cm long. The male presents yellow caudal, dorsal and anal fins that can become orange in favourable environmental conditions and good food availability. The body is greenish-brown with 10–12 transversal brown bands interspersed with thin silver bands, and in the bigger individual, a black-band on the caudal fin can appear during the reproductive period; the female is less colourful, presenting a grey body and 11–17 vertical bands (black or dark brown) along with both sides of the body (Leonardos and Sinis 1998; Cavarro *et al.* 2013; Facca *et al.* 2020). The species is omnivorous, mainly benthivores (Leonardos 2008; Facca *et al.* 2020). Although it can tolerate a wide range of salinity conditions, from 10 to 180 PSU, it prefers brackish or hyperhaline water bodies, subjected to tidal excursion and with strong fluctuations in chemical or physical parameters such as pH, turbidity, temperature, and salinity (Triantafyllidis *et al.* 2007; Cavarro *et al.* 2014). The species has an extended breeding season, from March to August (Leonardos and Sinis 1998). During the courtship, the male displays a range of aggressive behaviours to exclude the competitors following specific fixed action patterns; once ready it pushes the female to suitable spots for spawning, occasionally gently hitting the female belly to stimulate laying; hatching occurs ~14 days after the spawning, when the tide is favourable for the juveniles to move towards shallow isolated water, to decrease the risk of predation; the eggs do not hatch at the same moment but mostly singularly or in a small group (Triantafyllidis *et al.* 2007; Cavarro *et al.* 2013, 2014; Facca *et al.* 2020). The lack of larval dispersion and the preference of isolated habitats coupled with bottleneck effect might reduce the gene flow, thus leading to genetic divergence among populations (Maltagliati 2002; Leonardos 2008; Facca *et al.* 2020).



Fig. 2. Male of *Aphanius fasciatus* (right) and female of *A. fasciatus* (left).

Field survey on male population

In the context of an investigation on the population structure of the species, a large number of males were caught across the breeding season of 2021 from July to November in an artificial creek located in the Venice lagoon (45°28'31.7"N, 12°15'42.0"E) that was known to act as a breeding ground on the basis of previous observations. Fish were sampled lowering four small rectangular fyke nets (25 × 25 × 45 cm, mesh size 0.5 cm) with a metallic frame and a circular hole entrance of 65 mm at both sides to the creek bottom. After a sampling effort of 3 h, the content was investigated, and all the *A. fasciatus* individuals were placed in a plastic tray containing a millimetric laminated sheet to reference the fish measurements. All the fish were photographed for subsequent total-length measurements allowing the quick release to the wild. The measurements were conducted using the free software *ImageJ* (ver. 1.51, W. S. Rasband, US National Institutes of Health, Bethesda, MD, USA, see <https://imagej.nih.gov/ij/>; Schneider *et al.* 2012). The occurrence of the male vertical band was assessed by visually inspecting the size-frequency histograms of the male population over time.

Behavioural investigation under aquarium conditions

Sampling and housing

A subsample of mature individuals of *A. fasciatus* with evident sexual dimorphism and breeding colouration were collected in the sampling site mentioned in section *Field survey on male population* and transported to the Ca' Foscari University laboratory of zoology by using baskets with aerators. The transportation lasted less than 20 min, and the number of individuals transported to the laboratory was as low as possible to minimise impacts on the overall population. At the end of the experiments, the individuals were released in the above-mentioned sampling site. Once in the laboratory, individuals were housed in eight 125-L tanks set up at 30 PSU with artificial salt water (Prodac Ocean Fish OC30KG), which was the mean salinity measured *in situ* at the sampling site. Males were visually sorted by size and by the presence or absence of the black band on the caudal fin. In each holding tank, females were separated from males by a plastic septum that prevented fish from interacting but allowed water and hormones to circulate. This type of housing was chosen to maintain high hormone levels and to have individuals ready for mating. In the laboratory, the photoperiod regime was automatically reproduced following the natural cycle for the latitude and time of the year (May 2019, 45°28'41.7"N, 12°15'21.0"E). Temperature ranged from 24 to 26°C. Before proceeding with the analysis, the individuals were given 2 days of acclimatisation and, in the meantime, were fed twice a day *ad libitum* by Prodac Biogran Small BS250. The sidewalls of each tank were covered with thick, dark blue-coloured paper so as to prevent fish from excessive stress or conditioning.

Experimental apparatus

Four smaller experimental aquariums (40 L) were set up with a square tile (20 × 20 cm) covered with artificial green turf to recreate an ideal habitat for spawning (Cavraro *et al.* 2014). The bottom of each aquarium was covered with sand and the sidewalls were covered with dark paper similar to that used for the housing tanks to minimise visual disturbance. The experimental groups (whose composition is described below) were transferred to the experimental aquariums from the community tanks at the onset of each experiment (video-recording session) by means of a small aquarium net, and the group was substituted with a new group after each recording session.

Experimental design and procedure

Male–male competition and male tactics were investigated both in qualitative and quantitative terms. The apparatus was set up simulating a competitive or courtship arena where two males, differing or not in the presence or absence of the vertical black band on the caudal fin could have free access to a receptive female. Four groups were obtained to test male competition between males with (1) two males showing the black band (named group ‘vv’), (2) only one male showing the black band (named group ‘vx’), (3) no males showing the black band having body size above 30 mm (named group ‘xx’), and (4) no males showing the black band having body size of <30 mm (named group ‘xz’). Because the black band occurred above 35 mm in the male population (see Results), the size range of the two males and the size difference between the two males differed among the groups, according to the size structure of the natural population (Table 1). Only females with a highly pronounced belly were chosen to incentivise males to perform mating rituals. Concerning the male total length and the difference in size between the two tested males, no statistically significant correlation was found between the size and analysed variables (see Results). The behaviour of each group of fish was recorded with a digital camera (Canon Legria HF S30) positioned behind a U-shape box to minimise the disturbance during the trials, as proposed by Malavasi *et al.* (2010), focusing on the reproductive and aggressive behaviours of the breeding group. Once the group was released in the experimental tank, the light was turned on and the fish were given 30 min to acclimate.

Table 1. Group average total length (females and the two males analysed) of the four experimental groups tested (vv, vx, xx, xz) and the difference in size between the two males (Delta).

Trial	Female (mm)	Male 1 (mm)	Male 2 (mm)	Delta (mm)
vv	51.53	39.85	39.93	2.93
vx	39.06	42.13	27.32	14.8
xx	45.73	33.53	35.75	2.68
xz	49.13	24.65	28.68	0.66

After the acclimatisation, the camera was activated and the recording lasted 60 min. In total, 16 replicates were obtained, that is four replicates within treatment groups. On conclusion of the recording sessions, the fish were photographed and measured using *ImageJ* software.

Behavioural observations and data analysis

According to the behaviours previously described by Malavasi *et al.* (2010), in the present study the behavioural repertoire of male groups (Table 2) was assessed in terms of a number of acts, measured as either duration or frequency. To assess the overall spawning performance, the total number of spawning of each trial (TS) was also considered. Data were collected manually from watching the videos and analysed within the R statistical environment (ver. 3.6.0, R Foundation for Statistical Computing, Vienna, Austria, see www.r-project.org).

To explore the relationship between response variables and predictors, a generalised linear model was performed (GLMs; McCullagh and Nelder 1989). Each response variable was independently modelled using the most suitable distribution family and selecting the best model on the basis of the Akaike information criterion (AIC). Pairwise comparisons corrected for multiple testing (Holm adjustment) were performed when results of the fitted model were statistically significant. Six response variables were evaluated (SC, MC, SS, MS, TS and AB), and the four experimental groups (trials) and the difference in size between the two males analysed (delta) were fitted as predictors (Table 3). Concerning the male size difference, the four experimental groups were compared by a non-parametric Kruskal–Wallis test ($\alpha = 0.05$) followed by a pairwise comparison (Dunn’s test).

Results

Field survey of male population

In total, 4650 individuals were caught, of which 2765 were females and 1885 males. Females (mean size 31.44 ± 6.37 mm) tended to be larger than males (mean size 28.74 ± 5.25 mm), according to the sexual dimorphism of the species. Females ranged from 16.38 to 62.32 mm, whereas males from 15.96 to 56.03 mm. Of 1885 males, only 108 showed the caudal black band, which is expressed only in individuals bigger than 30 mm and especially in individuals over 35 mm. No black-banded males were caught from September. Fig. 3a shows the population of males by length class (1-mm intervals), and black-banded males are highlighted. The 1-mm length class was chosen to understand accurately at which size the black band appears. Fig. 3b shows the male population every fortnight, showing that the number of black-banded males decreased over time till week 38

Table 2. Description of the five behaviours analysed for *Aphanius fasciatus* and the unit measure.

Behavioural state	Abbreviation	Description
Single courtship	SC	Involves all the behaviours, performed by a single male, forming the courtship sequence as described by Malavasi <i>et al.</i> (2010).
Multiple courtship	MC	Involves the same behaviours described in SC but performed simultaneously by more than one male.
Single spawning	SS	S-shape posture associated with simultaneous quivering of the posterior part of the body by both male and female (Malavasi <i>et al.</i> 2010). Involving a single male.
Multiple spawning	MS	S-shape posture associated with simultaneous quivering of the posterior part of the body by both male and female (Malavasi <i>et al.</i> 2010). Involving more than one male.
Total spawning	TS	Total number of spawning (single spawning and multiple spawning).
Aggressivity	AB	Involves all the behavioural acts forming the aggressive sequence described by Malavasi <i>et al.</i> (2010) between two or more males.

Table 3. List of the generalised linear models fitted with the corresponding distribution family.

Label	Model structure	Distribution
m1	SC ~ Trial + Delta	Negative binomial
m2	MC ~ Trial + Delta	Gaussian
m3	SS ~ Trial + Delta	Gaussian
m4	MS ~ Trial + Delta	Gaussian
m5	TS ~ Trial + Delta	Gaussian
m6	AB ~ Trial + Delta	Negative binomial

SC, single courtship; MC, multiple courtship; SS, single spawning; MS, multiple spawning; TS, total spawning; AB, aggressive behaviours; trials, analysed groups; delta, male size difference.

(the first week of September), where no males showed the secondary sexual trait any longer.

Behavioural investigation under aquarium conditions

All experimental males exhibited, although with variable levels, all behaviours measured, including SC, MC, SS, MS and AB, with the exception of the 'vx' group, where no aggressive behaviour was recorded. The size difference in males among the experimental groups (delta) differed significantly between 'vx' and 'xz' (Fig. 4). The 'vx' presented high variance because the black band appears only in big individuals, as shown in the 'Field survey of male population' section of Results.

There is a tendency for a shorter duration in single courtship in the 'vv' group (two males with the black band), but there was no significant relationship between the analysed groups and the response variable, as well as no significant effects of the male size difference (delta) on the time spent in single courtship (Table 4, Model m1). The time spent in SC in the 'xz' group (two small individuals without band) was comparable to that in the other groups, showing the same reproductive repertory as for bigger individuals (Fig. 5a). Although the GLM showed a

significant relationship between 'trial' and 'multiple courtship (MC)' (Fig. 5b), with the 'vx' group engaging in paired courtship less frequently than did the 'vv' group, the pairwise comparisons did not show any significant differences between the groups (Table 4, Model m2). Additionally, no significant effect of delta on MC was found. The median duration of MC in the 'vx' group (one male presenting the black band and one without it) was the lowest, because the banded male (also bigger in size) tended to court more and monopolise the female.

No statistically significant effect of the variable 'trial' was found for single spawning and multiple spawning (Fig. 5c, d) as well as no significant effects of the male size difference on the response variables (Table 4, Models m3 and m4). Nevertheless, 'vx' group showed the lowest level of multiple spawning according to the time spent in multiple courtship as described above.

The frequency of aggressive behaviours differed significantly among the groups (Table 4, Model m6), with a positive significant relationship being found for 'trial' and 'AB', with the group 'vv' showing the higher values, and the group 'vx' showing no aggression (Fig. 5f). Groups 'vv' (consisting of two males with a black band) exhibited significantly higher frequencies of aggressive behaviours than did both the 'vx' and 'xz' groups, and the 'xx' group exhibited higher aggressive behaviours compared to the 'xz' group. Group 'vx' also showed significantly lower frequencies of aggressive behaviours than did 'xx'. Additionally, there was no statistically significant correlation between the size difference among the males and the frequency of aggressive behaviours (AB) (Spearman's rank correlation, $P > 0.001$). However, aggressive behaviours decrease when male size differences increase, reaching the highest frequency when male sizes are comparable (Fig. 6).

A highly statistically significant correlation was found between multiple courtship and aggressive behaviour (Spearman's rank correlation: $P < 0.001$) as well as between multiple spawning and aggressive behaviours (Spearman's rank correlation: $P < 0.01$), meaning that aggression increased when two males court or spawn in pairs (Fig. 7).

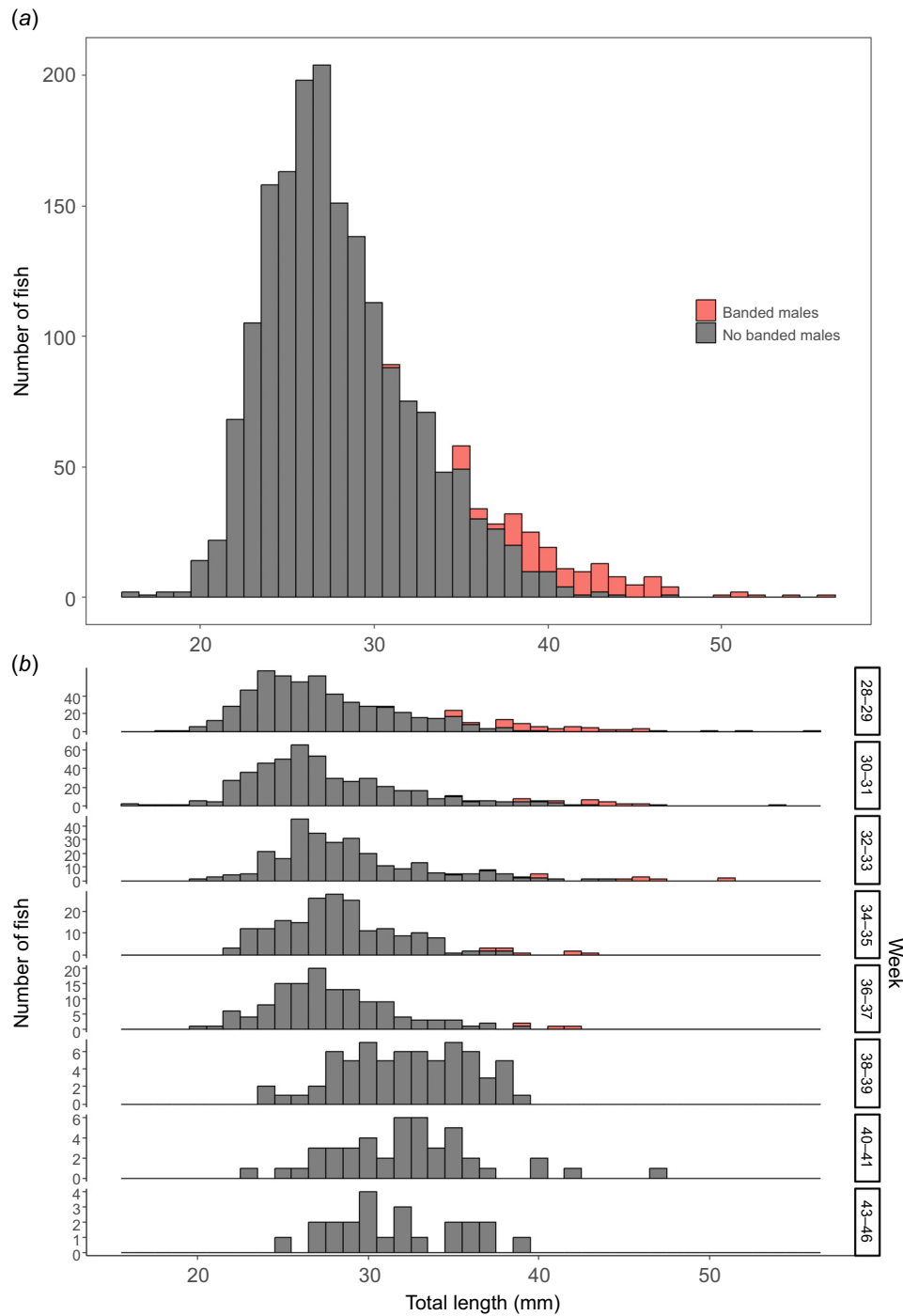


Fig. 3. (a) Length–frequency distribution of the sampled *Aphanis fasciatus* male population, according to 1-mm size-class intervals. Individuals showing the phenotypic trait ‘black band’ on the caudal fin are highlighted in red. (b) Length–frequency histogram, for every fortnight, of the sampled *Aphanis fasciatus* male population, according to 1-mm size-class intervals. Individuals showing the phenotypic trait ‘black band’ on the caudal fin are highlighted in red. Sampling started in Week 28 of 2021 (5 July 2021) and ended in Week 46 of 2021 (10 November 2021).

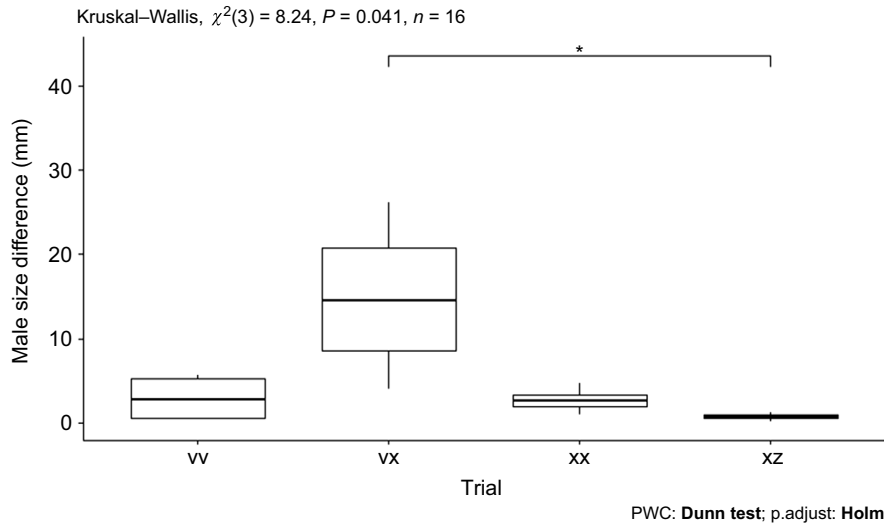


Fig. 4. Boxplot of males size difference between the analysed groups (mm). Differences between groups have been tested by performing a Kruskal–Wallis test followed by pairwise comparison (PWC) applying the Bonferroni correction. Significant differences are marked by an asterisk (*). The trial represents the analysed groups (vv = two males with black band on the caudal fin; vx = one male with black band on the caudal fin and one male without black band; xx = two males without black band on the caudal fin, having body size above 30 mm; xz = two males without black band on the caudal fin, having body size below 30 mm).

Table 4. Result of the six GLMs performed with pairwise comparisons (response variables: m1, single courtship; m2, multiple courtship; m3, single spawning; m4, multiple spawning; m6, aggressive behaviours).

Model	d.f.	Trial P-value	Delta P-value	Pairwise comparison	Predicted value
m1	15	>0.05	>0.05		
m2	15	<0.01	>0.05	vv vs vx: $P = 0.041$	1818.63
m3	15	>0.05	>0.05		
m4	15	>0.05	>0.05		
m5	15	>0.05	>0.05		
m6	15	<0.0001	>0.05	vv v. vx: $P = 0.029$	63.98
				vv v. xz: $P = 0.039$	58.82
				xx v. xz: $P = 0.045$	50.49

No statistically significant correlation was found between single courtship, single spawning and aggressive behaviours.

The overall spawning performance (TS) did not differ significantly among the analysed groups (Fig. 5e) and no significant relationship was found between predictors and the response variable (Table 4, Model m5).

Ethics

The care and use of experimental animals complied with Italian animal welfare laws, guidelines and policies as approved by the Article 18 of the Italian regional decree number 54/2012. All the experimental activities were supervised by a certified veterinarian with expertise in

captivity animal welfare (assignment REP 286/2022 PROT 74099 del 29/07/2022).

Discussion

The results of this study have provided further support that *A. fasciatus* mating system is based on a strong sexual selection and high competition among males and suggest the following four main conclusions: (1) the vertical band depends on an ontogenetic pattern, (2) courtship, spawning and aggressive behaviours are expressed by all the mature males irrespective of the presence or absence of the vertical band and the size, (3) sexual maturity occurs at least in fish of at least 20 mm long, with all males adopting the same reproductive strategy, being expressed in both female monopolisation attempt and alternative tactics (multiple spawning); and (4) large size and presence of the black band seem to contribute to male dominance. The attempt of a male to monopolise a female through fixed-pattern action is often disturbed by other males that can both participate in the courtship activities or act as a sneaker fertilising the eggs. The mating system appears to be polygynandric, and the multiple courtship often leads to aggressive behaviour when size difference between the two competing males is small. The presence of the black band on the caudal fin in only one of the two males, which often coincides with a large size difference, seemed to increase the chance of monopolising the female, reducing the multiple courtship

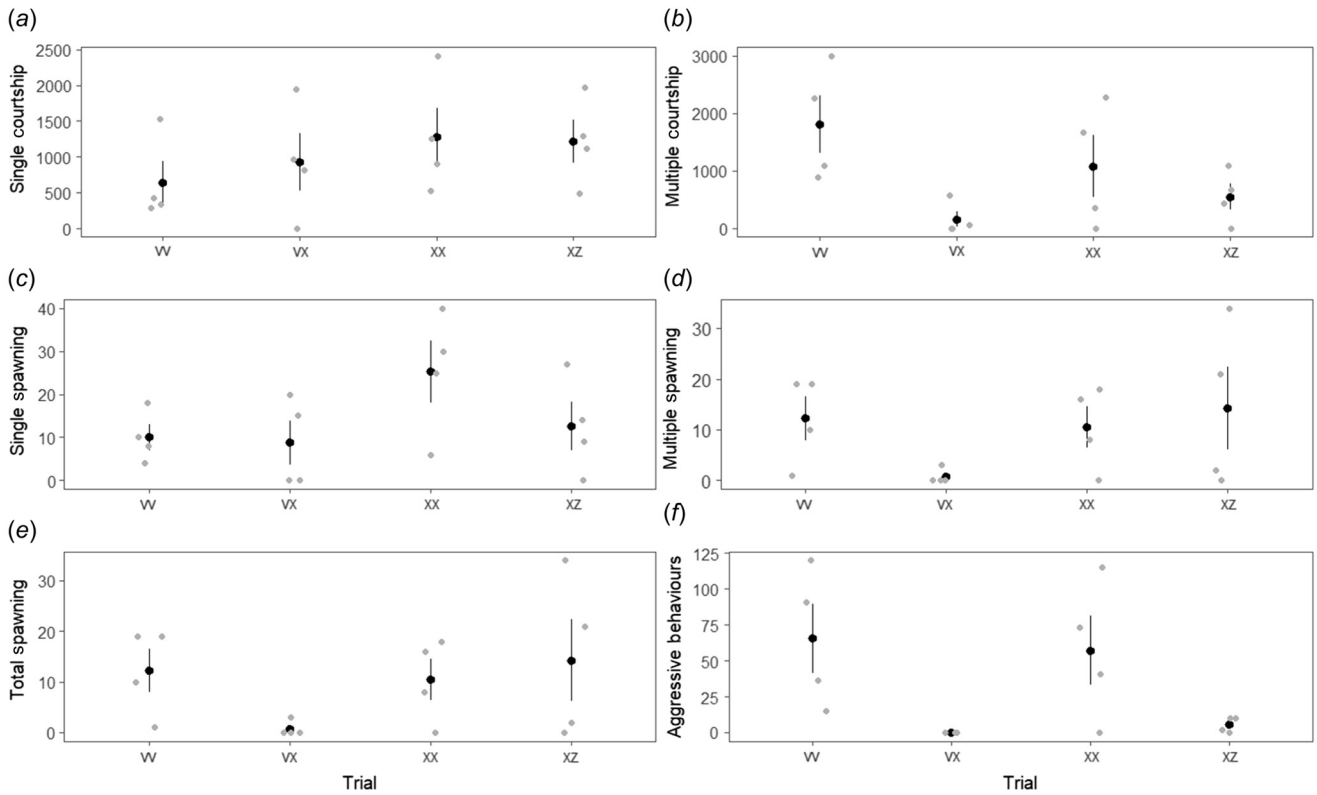


Fig. 5. (a) Plot of single courtship in terms of duration (seconds per hour) of *Aphanius fasciatus*. (b) Plot of multiple courtship in terms of duration (seconds per hour) of *Aphanius fasciatus*. (c) Plot of single spawning in terms of frequency (number of events per hour) of *Aphanius fasciatus*. (d) Plot of multiple spawning in terms of frequency (number of events per hour) of *Aphanius fasciatus*. (e) Plot of aggressive behaviours in terms of frequency (number of events per hour) of *Aphanius fasciatus*. (f) Plot of total spawnings in terms of frequency (number of events per hour) of *Aphanius fasciatus*. Mean value and standard error are highlighted. The trial represents the analysed groups (vv, two males with black band on the caudal fin; vx, one male with black band on the caudal fin and one male without black band; xx, two males without black band on the caudal fin, having body size of >30 mm; xz, two males without black band on the caudal fin, having body size of <30 mm).

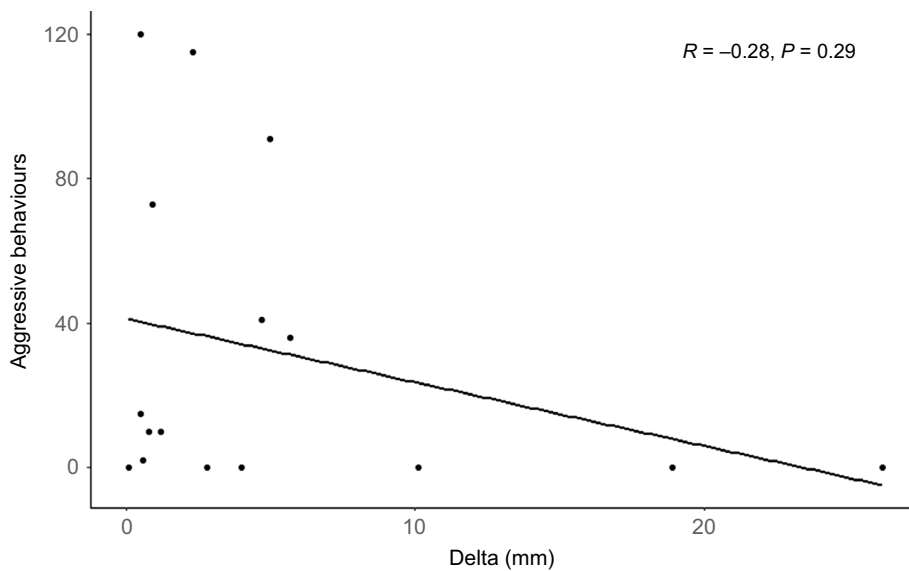


Fig. 6. Relationships between aggressive behaviours (number of events per hour) and the male size difference (delta) of *Aphanius fasciatus*.

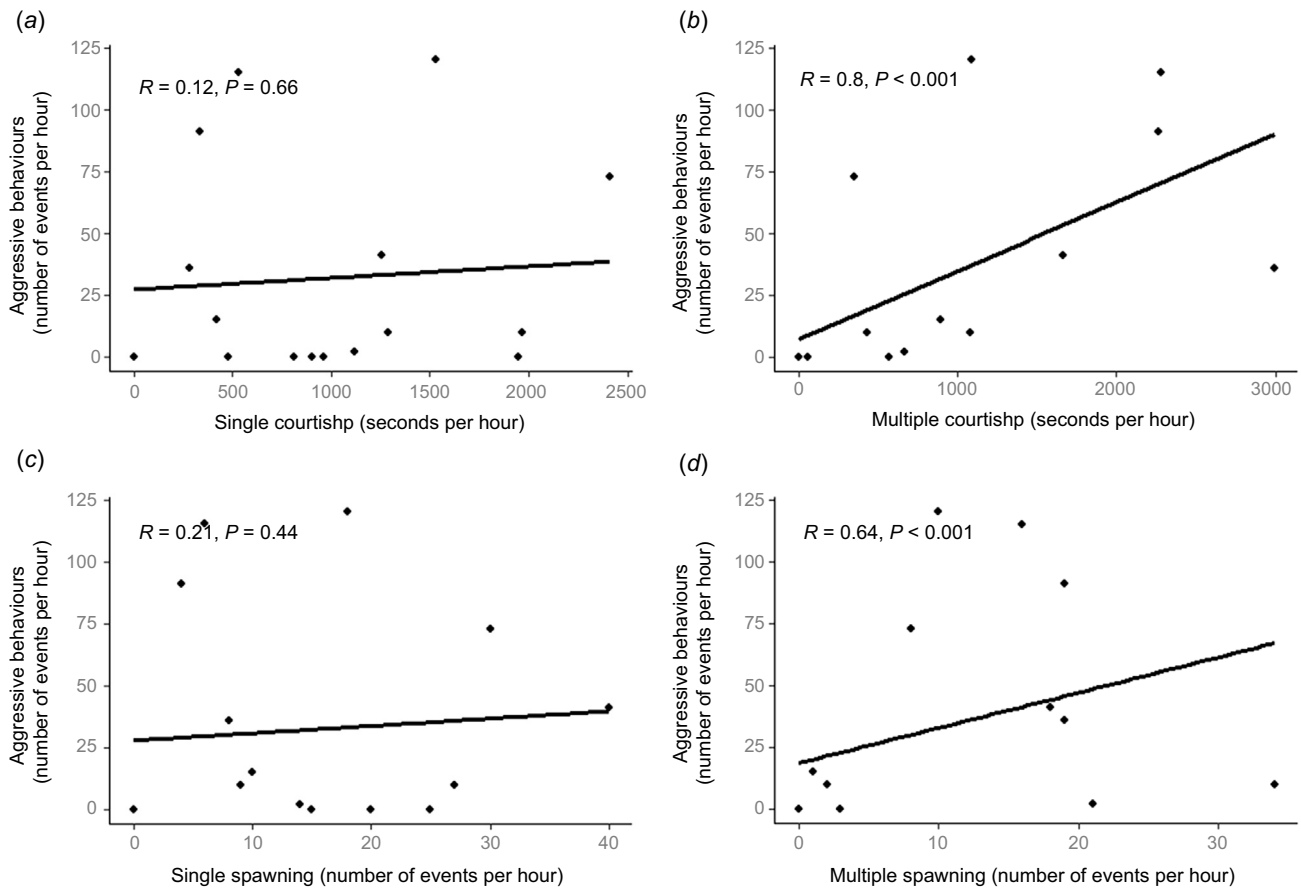


Fig. 7. Relationship between (a) single courtship and aggressive behaviours, (b) multiple courtship and aggressive behaviours, (c) single spawning and aggressive behaviours, (d) multiple spawning and aggressive behaviours of *Aphanius fasciatus*. Spearman rank correlation coefficient and P -value are showed.

behaviour and the aggressive interactions among males. However, the overall spawning performance (number of total spawnings) did not differ significantly among groups. Small size group (xz) showed low aggression and performed a range of alternative tactics such as multiple courtship, multiple spawning and sneaking that allowed these individuals to spawn with a frequency that is not different from that of larger males. By contrast, fish of either bigger size or showing the black band ('vv' and 'xx' groups) involved in multiple courtship behaviour, increased the frequency of aggressive interactions (Fig. 5f). Large-sized banded males appear to be fully dominant and capable of monopolising the females, especially when competing with smaller, non-banded males, as observed in group 'vx'. In these groups, levels of multiple courtship, multiple spawning and aggressive behaviour were lower than in other trials. The behavioural analysis suggested that the black band on the yellow–orange caudal fin is related to the number of aggressive behaviours, which are higher in the experimental Group 'vv', showing that these fish might be bolder and more motivated to monopolise the females. The scientific literature supports this hypothesis, namely that the caudal

fin colour is related to intimidatory functions (Barlow 1961; Kodric-Brown 1998; Cavararo *et al.* 2018) and the bright nuptial colour is important in agonistic interactions (Evans and Norris 1996; Kodric-Brown 1998). Bright colours are also signals of the capability to acquire and defend good foraging territory because the nuptial colouration is mainly related to carotenoid-based pigments, which are scarce in many environments (Kodric-Brown 1989; Frischknecht 1993). Melanic colouration was also associated to aggression in fish (Horth 2003; Almasi *et al.* 2008; Price *et al.* 2008) and non-aquatic species (Mafli *et al.* 2011). The black band, therefore, might appear in bigger and highly motivated individuals that manifest more aggressiveness with a greater likelihood of monopolising the female, also owing to its intimidating action that deters other males from entering into competition. However, only a small fraction of sampled males displayed the black band phenotypic trait. Habitat characteristics could influence the number of individuals capable of manifesting this secondary sexual trait, which could remain latent in environments with high predatory pressure or increase the mortality of individuals that invest more in sexual traits (Houde 1987; Gunderson 1997; Sowersby *et al.* 2022).

Archard and Braithwaite (2011) showed that fish caught in low-predation environments were bolder and more aggressive than those caught under high predator pressure. Thus, the distribution and occurrence of the investigated sexual trait within the population might be an indicator of habitat stressors such as predator presence, namely, the black-band being related to individuals' aggressiveness. Hormones might also drive the distribution of the black band within the population because 11-keto-testosterone (OT) has been found to peak in the reproductive period in many fish species, for instance, *Fundulus heteroclitus* (Mayer et al. 1990). Moreover, 11-keto-testosterone (OT) concentrations were correlated with secondary sexual traits and reproductive behaviour in stickleback males, showing that this androgen plays a key role in the reproductive repertory (Mayer et al. 1990). Brantley et al. (1993) showed that, like melanin colouration, high OT concentrations were correlated with size, sexual traits, aggressiveness and dominance in teleost fishes. Similarly, the caudal black band in male *Aphanius fasciatus* could be a melanin-dependent trait under the control of 11-keto-testosterone. Further studies could test this hypothesis.

In conclusion, this study has highlighted that the Mediterranean killifish, *Aphanius fasciatus*, is an excellent model for studying a polygynandrous mating systems, on the basis of intense male–male competition. From a size of 20 mm, males adopt the same strategy, based on female monopolisation attempt and multiple courtships and spawnings, with levels of aggression, competitive ability and dominance being increased with a larger a size and the expression of a melanin trait (the black band). Although no statistically significant correlation was found between male size differences and the analysed variables, further experiments comparing size-matched males might better disentangle the effects of body size and band presence. Further research should investigate how habitat constraints and environmental stressors drive the distribution of the secondary sexual trait 'black band' among the populations, the possibility of using this trait as an indicator of habitat stressors, and whether fish showing the black band can reduce sperm competition monopolising the females.

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