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Research Paper

Behavioral and physiological dynamics of a native minnow exposed to invasive fish species

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ABSTRACT

The introduction of invasive alien species is a major threat to freshwater biodiversity, particularly in Mediterranean ecosystems where seasonal droughts increase population density and social stress. This study investigated the effects of the presence and of variables densities of two widespread invasive fish species *Gambusia holbrooki* (Girard, 1859) and *Lepomis gibbosus* (Linnaeus, 1758), on the behaviour and physiology of the Iberian endemic *Squalius alburnoides* (Steindachner, 1866). Under baseline density, exposure to *G. holbrooki* significantly altered the behaviour of *S. alburnoides*, leading to increased aggression towards conspecifics, enhanced evasion, and a rise in the number of attacks suffered. These behavioral changes were accompanied by a reduction in forebrain dopamine levels, suggesting that dopaminergic influence stress-related responses. In contrast, interactions with *L. gibbosus* under the same conditions did not produce significant behavioral or physiological effects, although conspecific aggression showed temporal fluctuations. Under high-density conditions, both invaders intensified antagonistic interactions with *S. alburnoides*. Significant neurochemical alterations occurred solely in fish exposed to *G. holbrooki*, which showed elevated 5-HIAA concentrations and increased DOPAC/DA and 5-HIAA/5-HT ratios, indicating activation of serotonergic and dopaminergic pathways. Unexpectedly, plasma cortisol levels in *S. alburnoides* decreased in the presence of both invader species, suggesting a possible downregulation of the hypothalamic–pituitary–interrenal axis under putative social stress. Overall, our results demonstrate that invasive species differentially modulate behavioral and physiological stress responses in *S. alburnoides*, with *G. holbrooki* exerting stronger effects than *L. gibbosus*, and that higher densities amplify these species-specific interactions.

1. Introduction

The introduction of invasive species represents one of the main threats to aquatic ecosystems, causing profound disruptions to ecological interactions and contributing to biodiversity loss [1]. These species, typically introduced through human activities, are able to establish and multiply, with significant ecological, economic, and social impacts [2–4]. Globalization, along with the increase in transportation and international trade, has facilitated their spread, often exacerbated by a lack of effective regulation [5,6]. Recent projections indicate that, in the absence of effective control measures, the number of invasive species in Europe is expected to increase by approximately 64% by 2050, corresponding to an additional 2543 alien species, making Europe the continent with the highest relative and absolute increase worldwide [7].

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Freshwater ecosystems are particularly vulnerable to biological invasions, and teleost fish are amongst the most affected [8,9]. An extreme case occurs in temporary rivers, in which the interruption of flow during dry months leads to the formation of small pools, with fish becoming concentrated in a limited space [10]. This results in increased population densities and competition for resources, which is further exacerbated by the presence of invasive species. Such conditions can intensify aggression, reduce growth, and cause chronic stress [11-13]. The specific composition of these groups, particularly the ratio of native to invasive species, can further modulate the intensity and conflicts of these interactions [14]. However, the outcome of social interactions under experimental conditions may also depend on individuals' prior social experience in the wild, including population density, habitat structure, and familiarity with conspecifics. Such factors are difficult to control when working with wild-caught fish and may contribute to interspecific differences in behavioral plasticity and stress responsiveness.

Stress responses in fish involve multiple levels of physiological organization. At a primary level, activation of the hypothalamic–pituitary–interrenal (HPI) axis leads to the release of cortisol, a hormone essential for coping with adverse situations [15]. Simultaneously, catecholamines such as adrenaline, noradrenaline, and dopamine are released, triggering rapid responses including elevated heart rate and blood circulation [16]. These responses are accompanied by changes in the release of brain neuromodulators, such as serotonin and dopamine, which are closely linked to behavior regulation and adaptation to different stress intensities [17–20].

Stress directly influences fish behavior, affecting feeding, reproduction, and social interaction patterns [15,16]. In altered environments, such as those invaded by exotic species, native individuals tend to adopt more evasive or cautious behaviors [21,22]. The presence of invaders can force native species to relocate to suboptimal habitats, reducing access to resources and compromising reproductive success [4,23,24]. Social behaviors such as aggression and competition thus become sensitive indicators of ecological disturbance, being strongly modulated by stress factors and interspecific interactions [16,25,26].

Despite the well-documented ecological impacts of invasive fish species on native freshwater communities, important knowledge gaps remain regarding the behavioral and physiological mechanisms underlying these effects. Most studies on invasives have traditionally focused on changes in species distribution, abundance, or population-level traits, while comparatively fewer have addressed how invasive species alter social interactions, aggression dynamics, and stress physiology of native fishes in laboratorial conditions [4,24]. Experimental evidence integrating behavioral responses with neuroendocrine and monoaminergic regulation is particularly scarce, especially for European freshwater systems and endemic species. Moreover, the role of social context, such as population density and species composition, as mediators of native fish responses to invasive competitors remains poorly understood [14]. Addressing these gaps is essential to better link individual-level responses to broader ecological consequences of biological invasions.

This is the case of the interactions among the invasive species *Gambusia holbrooki* (Girard, 1859) and *Lepomis gibbosus* (Linnaeus, 1758), and the native *Squalius alburnoides* (Steindachner, 1866) a species widely distributed in Iberian rivers of the Atlantic drainage, which together constitute a valuable ecological model for investigating the effects of invasive species presence and density in Mediterranean freshwater ecosystems. Both *G. holbrooki* and *L. gibbosus* are among the most widespread invasive freshwater fish species globally and are listed as invasive alien species of Union concern under EU Regulation 2019/1262 [27]. They are characterized by high environmental tolerance, behavioral plasticity, and aggressiveness, and have been associated with negative impacts on native ichthyofauna [28,29]. In contrast, *S. alburnoides* is an endemic leuciscid of the Iberian Peninsula, ecologically important and potentially vulnerable to pressure from such highly competitive invaders. Due to its small-bodied, fusiform body shape characteristic of cyprinid fishes, reduced body size, and gregarious habits—traits shared with many other threatened freshwater species—*S. alburnoides* is an ideal model for assessing the behavioral and physiological impacts of biological invasions [30]. This experimental framework allows the investigation of how invasive species employing distinct ecological strategies can affect multiple levels of response in native fish.

Both invasive species considered in this study are known to exert strong competitive pressure on native fish through aggressive behavior and resource exploitation. *G. holbrooki* is highly aggressive and efficient in food acquisition, often displacing native species from feeding areas and preying on fish larvae and aquatic invertebrates, thereby altering community structure and recruitment dynamics [29,31]. Similarly, *L. gibbosus* is a highly adaptable and opportunistic species that competes effectively for food and shelter, while exhibiting pronounced interspecific aggressiveness that should intensify competitive interactions with native fishes [28]. These traits are expected to place smaller-bodied, gregarious native species such as *S. alburnoides* at a competitive disadvantage, particularly under conditions of elevated density and spatial restriction.

From a behavioral perspective, sustained exposure to aggressive and dominant invaders may promote submissive or avoidance strategies in native fish, reducing access to resources and increasing chronic stress levels. Such shifts in social behavior have been documented in native species confronted with invasive or novel competitors, often accompanied by altered stress physiology and neuroendocrine regulation [16,21,22]. In this context, increased aggression by invasive species is expected to reshape social hierarchies within mixed groups, potentially leading to elevated stress responsiveness and neurophysiological adjustments in *S. alburnoides*. Understanding these mechanisms is particularly relevant in Mediterranean freshwater systems, where seasonal habitat contraction further intensifies competition and social interactions.

Here we aimed to assess the effects of the presence of the invasive species *G. holbrooki* and *L. gibbosus* on the behavior and physiology of the native species *S. alburnoides*, under both lower and higher-density conditions. Behavioral responses (aggression, submission) and physiological markers (cortisol levels and brain monoamines) were analyzed. We hypothesized that the presence of invasive species, particularly under higher-density conditions, would induce submissive behaviors in *S. alburnoides* and neurophysiological changes indicative of stress, including increased cortisol and alterations in dopamine and serotonin levels.

2. Material and methods

2.1. Species collection and maintenance conditions

Three freshwater fish species were used in this study: the native *S. alburnoides* and two invasive exotic species, *G. holbrooki* and *L. gibbosus*. Individuals of *S. alburnoides*, a cyprinid endemic to the Iberian Peninsula, were collected from the Vascão River, a tributary of the Guadiana River (37°24′30.52″N; 7°55′20.05″W), known for its high diversity of native fish [10,32]. *G. holbrooki* was sampled from Lago da Malagueira, an urban pond in Évora (38°34′7.46″N; 7°55′25.18″W), and *L. gibbosus* from a reservoir near the town of Redondo (38°39′48.25″N; 7°30′2.91"W), where established populations of both invasive species are present. Sampling locations were selected to ensure naïve individuals' representative of each native/invader species. *S. alburnoides* individuals were collected from a site where the presence of invasive species is relatively low, in order to minimize potential habituation effects; however, given the widespread distribution of *G. holbrooki* and *L. gibbosus* in Mediterranean river systems, prior exposure to these species cannot be entirely ruled out. Additionally, *S. alburnoides* is absent from the locations where individuals of each invasive fish species were collected. No quantitative estimates of local population densities were available for either of the lentic systems where invasive species were

captured. However, *G. holbrooki* shares the lake with three other invasive fish species: *Ameiurus melas* (Rafinesque, 1820), *Cyprinus carpio* (Linnaeus, 1758), and *Australoheros facetus* (Jenyns, 1842). In contrast, in the waterbody where *L. gibbosus* was collected, only one other invasive species was present, *C. carpio*. Regarding the Vascão River, there is some information on the fish community in which *S. alburnoides* occurs, indicating that this species is one of the most abundant, although with large variations among sites and throughout the year (see, for example, [33]).Therefore, individual social history and intraspecific experience prior to capture could not be standardized across species or sampling locations. This limitation is inherent to studies using wild-caught individuals and was considered in the interpretation of behavioral and physiological responses.

A total of 176 individuals of *S. alburnoides*, 88 of *G. holbrooki*, and 88 of *L. gibbosus* were collected between June 2023 and February 2024 (see below). *S. alburnoides* had an average standard length of 6.39 cm (SD = 1.15), *G. holbrooki* 4.06 cm (SD = 0.78), and *L. gibbosus* 5.71 cm (SD= 1.64). Sex was determined for *G. holbrooki*, which exhibits clear sexual dimorphism in adults; sex determination was not feasible for *S. alburnoides* and *L. gibbosus* due to the absence of external dimorphic traits in the sampled size range.

Fish were collected in field campaigns carried out in June, August, and October 2023 and February 2024. These periods partially overlap with the known reproductive seasons of the study species. In Mediterranean and Iberian freshwater systems, *S. alburnoides* typically reproduces from spring into early summer (March–July) [34], *G. holbrooki* exhibits reproductive activity primarily during warmer months from spring through autumn (approximately April–October) [35], and *L. gibbosus* generally spawns in late spring to summer (roughly May–July) [36].Therefore, although some captures occurred early or late relative to peak breeding windows, sampling did not coincide with the core reproductive peak of all species and likely included both pre- and post-reproductive individuals.

During fish collection environmental conditions (e.g., temperature, flow regime, and habitat structure) may differ among sampling sites, these parameters were not quantitatively recorded at the time of collection. Therefore, potential site-specific environmental variation could not be formally assessed and was considered as an inherent source of variability associated with the use of wild-caught individuals.

Fish were collected using electrofishing, minimizing impact on natural populations; however, quantitative estimates of catch per unit effort (CPUE) were not available. As a result, natural variation in local population density could not be formally quantified or incorporated into the experimental design.

Following electrofishing, individuals were immediately transferred to large containers (approximately 50 L) filled with site water and kept shaded whenever possible. Containers were left uncovered to allow passive gas exchange, and water was gently agitated periodically to promote oxygenation. Fish were transported to the laboratory within approximately 2–3 h for *S. alburnoides*, ~1 h for *L. gibbosus*, and <1 h for *G. holbrooki*. During transport, fish were not continuously monitored due to logistical constraints; however, individuals were checked whenever stops occurred, and no signs of acute distress or mortality were observed.

After capture, the fish were transported to the laboratory and housed in three 200 L tanks (100 × 50 × 40 cm) equipped with water recirculation and biofiltration systems. Each aquarium was divided into two 100 L compartments to separate experimental conditions. The fish were allowed to acclimate for five days under controlled conditions (12:12 h light/dark photoperiod, average temperature of 22°C, pH ≈ 8.00), with continuous monitoring of water quality (temperature, pH, and dissolved oxygen). Nitrogenous compounds (ammonia, nitrite, and nitrate) were also regularly measured to ensure they remained within acceptable and stable levels. In addition to biofiltration, approximately 25% of the water in each tank was renewed every two weeks during routine cleaning, which further prevented the accumulation of nitrogenous waste.

During the acclimation period, fish were monitored daily to assess general health and behavior. All individuals showed clear signs of acclimation prior to the onset of experimental trials, including active exploration of the tanks and regular acceptance of the provided food. Throughout both the acclimation period and the experiments, fish were fed a commercial granulated diet (D-Allio Plus, Tropical®) until apparent satiety, with most individuals actively feeding during each feeding event. No abnormal locomotor behavior, lethargy, or signs of distress were observed before experimental manipulation.

All animal handling and experimental procedures were conducted in compliance with European and national legislation for the protection of animals used for scientific purposes and were approved by the institutional animal welfare bodies ORBEA-UE and ORBEA-CIBIO-BIOPOLIS. Fish collection from the wild was done under license (License No. 320/2023/CAPT, Fishing Credential No. 39/2022) emitted by the Instituto da Conservação da Natureza e das Florestas (ICNF).

2.2. Experimental design

Behavioral trials were conducted between June 2023 and March 2024. After capture, fish were allowed to acclimate to laboratory conditions for five days before being transferred to the experimental aquaria. Each experimental block followed a standardized schedule: fish were introduced into 100 L aquaria and given 24 h of acclimation, after which six consecutive days of experimentation were carried out. Behavioral recordings took place on days 2, 4, and 6, while on day 7 the tanks were cleaned, and samples were collected for physiological analyses. Following each block, a five-day recovery interval was maintained to minimize cumulative stress before initiating the next round, after which the same procedure was repeated.

2.3. Experimental trials

Fish were distributed in 100 L aquaria under two treatment types: (i) control tanks containing only *S. alburnoides*, and (ii) mixed tanks composed of equal numbers of *S. alburnoides* and invasive individuals (*G. holbrooki* or *L. gibbosus*). In the first phase (baseline density), each aquarium housed eight fish: either eight *S. alburnoides* in control groups, or four *S. alburnoides* together with four invasive individuals in mixed groups. To ensure equivalent sample sizes of *S. alburnoides* across treatments, three independent replicates of the control condition and six replicates of the mixed condition were performed. This design enabled direct comparisons of *S. alburnoides* behavior between tanks with only conspecifics and those with invasive species present.

In the second trial, the effect of increased density on these interactions was tested. Each aquarium housed 16 individuals: either 16 *S. alburnoides* in control groups, or eight *S. alburnoides* together with eight invasive individuals in mixed groups. This setup simulated the ecological conditions observed in the Vascão River during summer, when fish are concentrated in small pools due to reduced water flow [10]. To balance the number of native individuals across treatments, two independent replicates of the control condition and four replicates of the mixed condition were performed. The same recording and behavioral analysis protocol was applied as in the baseline phase, allowing comparisons across densities while accounting for species composition effects.

2.4. Behavioral recordings

To minimize external disturbance, recordings were performed in the absence of observers in the aquarium room. A webcam was positioned in front of each aquarium to capture the widest field of view with minimal blind spots, while the connected laptop was placed behind a camouflaging curtain to prevent light interference. Each trial lasted 30 min and was conducted during the normal activity period of the fish to ensure the

recording of spontaneous behaviors.

Behavior was quantified from the video recordings using BORIS (Behavioral Observation Research Interactive Software; [37]). Three main categories of behavior were scored following Matthews and Wong [38] (Table 1): (i) aggressive behaviors, including chases and bites, recorded separately for conspecific and heterospecific interactions; (ii) submissive behaviors, namely escape responses, typically expressed during or after conflict; and (iii) summary variables, including the total number of attacks (chases + bites) and the total number of attacks suffered, the latter calculated from conspecific aggression plus heterospecific escape responses.

2.5. Physiological analysis: cortisol and brain monoamines

At the end of each experimental block, all fish were measured. For each species, half of the individuals were ethically euthanized via decapitation [39] and processed for physiological analysis: heads (including operculum) and brains were stored at −80°C and sent to the Technical University of Denmark (DTU) for cortisol and monoamine quantification. The remaining *S. alburnoides* were returned to the Vascão River, while individuals of invasive species (*Gambusia holbrooki* and *Lepomis gibbosus*) were euthanized by thermal shock, in compliance with institutional animal welfare guidelines. This procedure followed the Portuguese Decree-Law No. 113/2013, which regulate the protection of animals used for scientific purposes, as well as Regulation (EU) 1143/2014 concerning invasive alien species, which mandates humane euthanasia of invasive organisms for biosecurity and containment purposes.

Table 1
Table of *S. alburnoides*'s behaviors to be recorded. ε= Behaviour recorded only in mixed groups. All active behaviors listed correspond to actions performed by *S. alburnoides*. Adapted from Matthews & Wong [38].

Category	Behaviour	Description
Aggressive	**Chases**	Rapid movement of one individual towards another, typically followed by close pursuit. Can be conspecific when between two individuals of the *S. alburnoides* species, or heterospecific when *S. alburnoides* chases an individual from one of the invasive species.
	Bites	Physical contact in which an individual bites another often occurring during or following a chase. Can be conspecific when between two individuals of the *S. alburnoides* species, or heterospecific when *S. alburnoides* chases an individual from one of the invasive species.
Submissive	**Escapes**[ε]	Rapid movement of an individual away from another, typically in response to a perceived threat or aggressive approach. Can be conspecific when between two individuals of the *S. alburnoides* species, or heterospecific when *S. alburnoides* runs away from an individual from one of the invasive species.
Other	**Total number of Attacks** (Total Attacks)[ε]	Sum of all aggressive events (chases and bites). Can be conspecific when between two individuals of the *S. alburnoides* species, or heterospecific when *S. alburnoides* attacks individuals from one of the invasive species. Total Attacks= Chases + Bites (can be conspecific or heterospecific)
	Attacks suffered	Total number of attacks suffered by *S. alburnoides*. In control groups, this includes only conspecific attacks (chases + bites). In mixed groups, it includes both conspecific attacks and heterospecific escapes, which reflect avoidance responses to attacks by invasive species. Attacks Suffered= Conspecific attacks received + Heterospecific escapes (avoidance of invader aggression)

Gill tissue (all gill arches from the left side) was collected from fish head samples and homogenized in 500 μL of phosphate-buffered saline 150 mM, pH 7.4, by means of an ultrasonic homogenizer (Sonopuls HD3100, Bandelin Electronic, Germany). Homogenates were centrifuged (10,000 x g, 10 min) and gill cortisol levels were quantified from the samples using an enzyme-linked immunosorbent assay (ELISA, Tecan/IBL, RE52611), following the manufacturer's protocol. Cortisol data was normalized by the amount of soluble protein in the supernatants [17], which was quantified with a commercial kit (23227 Pierce™ BCA Protein Assay Kit, Thermo Scientific, Rockford, USA).

For monoamine analysis, the forebrain of each individual (comprising the telencephalon and the olfactory bulb) was dissected out from the still-frozen head for the quantification of dopamine (DA), serotonin (5HT), and their oxidative catabolites: DOPAC (3,4-dihydroxyphenylacetic acid) and 5HIAA (5-hydroxyindoleacetic acid). In addition, the ratios DOPAC/DA and 5HIAA/5HT were calculated (values expressed per unit of tissue mass). Samples were weighed and homogenized in 400 μL of perchloric acid solution (0.4 M PCA, 0.1 mM EDTA) using an ultrasonic homogenizer (Sonopuls HD3100, Bandelin Electronic, Germany), followed by centrifugation at 14,000 x g for 10 min at 4°C. The supernatant was diluted 1:3 in the same solution and analyzed using high-performance liquid chromatography with electrochemical detection (HPLC-EC), employing a Supelcosil LC-18-DB column (15 cm × 4.6 mm, 5 μm) (Merck, Darmstadt, Germany), and a mobile phase consisting of 74 mmol L^{-1} NaH_2PO_4, 0.14 mmol L^{-1} EDTA, 0.54 mmol L^{-1} sodium 1-octanesulfonate, and 15.3% (v/v) methanol, with pH 3.0.). Electrochemical detection was performed using an ESA Coulochem II system equipped with a 5020 conditioning cell and a 5011A analytical cell with first and second electrodes set at +200 mV and -350 mV, respectively.

2.6. Statistical analysis

Analyses were conducted in R [40] using the packages *tidyverse* [41], *lme4* [42], *lmerTest* [43], *emmeans* [44], *car* [45], and *ez* [46].

Physiological data (plasma cortisol and monoamine concentrations) were analyzed using one-way ANOVA, Welch's ANOVA, or Kruskal–Wallis tests, depending on the distribution and homogeneity of the data. ANOVA was used when assumptions of normality and homogeneity of variances were met; Welch's ANOVA was applied when data were normally distributed but violated homogeneity of variance (as assessed by Levene's test); and the Kruskal–Wallis test was employed for non-normally distributed data. These tests were appropriate for physiological variables as they involved independent measures from different individuals, without repeated observations.

Behavioral data were analyzed using linear mixed-effects models (LMMs) to account for repeated measures across days and the nested structure of the dataset (i.e., individuals within tanks). Each model included Group (Control vs. Mixed) or Type (between Conspecifics vs. Heterospecifics) and Day (D2, D4, D6) as fixed effects, along with their interaction, and tank identity as a random intercept. Residual normality was further assessed post hoc through visual inspection of histograms and Q–Q plots and confirmed with Shapiro–Wilk tests. In cases where assumptions were not fully met but residual deviations were not severe, LMMs were retained due to their robustness to moderate violations and suitability for unbalanced designs. When residual distributions showed strong deviations from normality or group sample sizes were too small for reliable residual testing, non-parametric alternatives were used. Specifically, Wilcoxon signed-rank tests were used for paired comparisons between interaction types, and Friedman tests were applied for comparisons across days within repeated measures designs. Post-hoc pairwise comparisons following LMMs were performed using the emmeans package with Tukey adjustments. To account for the difference in the number of individuals between control (8 individuals) and mixed (4 individuals) groups, behavioral frequencies were normalized

by dividing the total number of behaviors recorded by the number of individuals present in each tank. Exploratory Spearman rank correlations were conducted to assess potential associations between behavioral and physiological variables, acknowledging the limited sample size in some experimental groups. These tests were chosen due to small sample sizes and occasional violations of normality, particularly under high-density conditions. Given the limited number of samples in the control groups, correlation analyses were restricted to the mixed treatment groups.

Sampling time within each experimental block was not included as an explicit factor in the statistical analyses, as physiological samples were collected following a standardized and consistent experimental schedule across treatments. However, as experimental blocks were conducted across different months, we performed additional exploratory analyses to assess potential temporal variation in physiological parameters. One-way comparisons across sampling periods were conducted using ANOVA, Welch's ANOVA, or Kruskal–Wallis tests depending on data distribution and variance homogeneity (see Supplementary Table S7). These analyses revealed significant differences among sampling periods for several physiological variables. Because sampling time was partially confounded with experimental treatments and densities, these tests were interpreted as indicative of natural temporal and seasonal variability rather than independent experimental effects. Consequently, sampling period was not incorporated as a fixed factor in the main models, but its potential influence was considered when interpreting physiological results.

An overview of the statistical tests applied to each behavioral and physiological parameter is provided in the corresponding tables referenced throughout the Results section, where test selection is reported alongside the respective results.

3. Results

3.1. Lower density conditions

Under baseline conditions, *S. alburnoides* displayed altered behavior in response to the presence of invasive species, with more pronounced effects in the presence of *G. holbrooki*. In mixed groups with *G. holbrooki*, *S. alburnoides* performed significantly more conspecific chases ($p = 0.0051$) and suffered more attacks ($p = 0.0023$) compared to control groups (Table 2, Fig. 1).

Within these mixed groups, no significant differences were detected between conspecific and heterospecific attacks ($p > 0.05$; Table 3, Fig. 2). Additionally, no significant differences were found between the number of attacks suffered and caused during heterospecific interactions ($p = 0.8438$).

In contrast, no significant behavioral differences were observed between control and mixed groups exposed to *L. gibbosus*. However, time-related effects emerged: the frequency of chases ($p = 0.0251$), bites ($p = 0.0137$), and total conspecific attacks ($p = 0.0211$) varied significantly across the three experimental days (Table 2, Fig. 3).

In mixed groups (exposed to *L. gibbosus*), conspecific chases ($p < 0.001$), escapes ($p = 0.019$), and total attacks ($p < 0.001$) were more frequent than heterospecific ones. Bites were also more frequent in conspecific attacks ($p = 0.0014$), with differences across days ($p = 0.0108$) and a significant interaction between interaction type and time ($p = 0.0168$) (Table 3, Fig. 4).

At the physiological level, only *S. alburnoides* exposed to *G. holbrooki* showed significant changes: forebrain dopamine (DA) levels were higher in control groups than in mixed groups (ANOVA, $p = 0.02$; Table 4; Fig. 5). No significant differences were found in plasma cortisol, DOPAC, serotonin (5-HT), 5-HIAA, or in the metabolic ratios DOPAC/DA and 5-HIAA/5-HT. In the *L. gibbosus* treatment, no significant physiological differences were observed between control and mixed groups; however, *S. alburnoides* exhibited a marginal decrease in the forebrain DOPAC/DA ratio in mixed groups ($p = 0.07$), reflecting a tendency toward decreased dopamine turnover (Table 4, Fig. 5).

Additional analyses revealed significant differences among sampling periods for all physiological variables (Supplementary Table S7), indicating temporal variation likely associated with seasonal and block-specific factors.

Table 2
Summary of statistical results for behavioral variables of *S. alburnoides* under baseline and high-density conditions. Linear mixed-effects models (LMMs) were used to assess the effects of Group (Control vs Mixed), Day (D2, D4, D6), and their interaction (Group × Day) on each behavior, accounting for tank identity as a random factor. Significant results are shown in **bold** ($p < 0.05$). Abbreviations: *G*= Group effect; *D*= Day effect; *G* × *D*= Group × Day interaction; ns= not significant.

† Total Attacks= Chases + Bites ε Attacks Suffered= Conspecific Chases + Heterospecific Escapes

Species	Condition	Behavior	Test	Group (G)	Day (D)	*G* × *D* Interaction
With *G. holbrooki*	Baseline	Chases	LMM	***0.0051***	0.1719	ns
		Bites	LMM	0.0649	0.1448	ns
		Escapes	LMM	***0.0051***	0.1459	ns
		Total Attacks†	LMM	***0.0055***	0.1607	ns
		Attacks Suffered ε	LMM	***0.0023***	0.1395	ns
	High-density	Chases	LMM	0.0648	0.2420	ns
		Bites	LMM	0.9119	0.9873	ns
		Escapes	LMM	0.0650	0.2471	ns
		Total Attacks†	LMM	0.0681	0.2587	ns
		Attacks Suffered ε	LMM	0.0634	0.7391	ns
With *L. gibbosus*	Baseline	Chases	LMM	0.4379	***0.0251***	ns
		Bites	LMM	0.5467	***0.0137***	ns
		Escapes	LMM	0.4234	***0.030***	ns
		Total Attacks †	LMM	0.4523	***0.0211***	ns
		Attacks Suffered ε	LMM	0.1394	0.1468	ns
	High-density	Chases	LMM	***0.0210***	0.4411	ns
		Bites	LMM	***0.0223***	0.9656	ns
		Escapes	LMM	***0.0210***	0.4411	ns
		Total Attacks †	LMM	***0.0200***	0.4397	ns
		Attacks Suffered ε	LMM	***0.0209***	0.4532	ns

When the effect of Day was significant, pairwise comparisons were conducted using estimated marginal means (emmeans) with Tukey adjustment. Significant pairwise differences were found between D4 and D6 for conspecific chases ($p = 0.0231$), escapes ($p = 0.0278$), and total attacks ($p = 0.0182$), and between D2 and D6 for conspecific bites ($p = 0.0150$), in the presence of *L.* gibbosus.

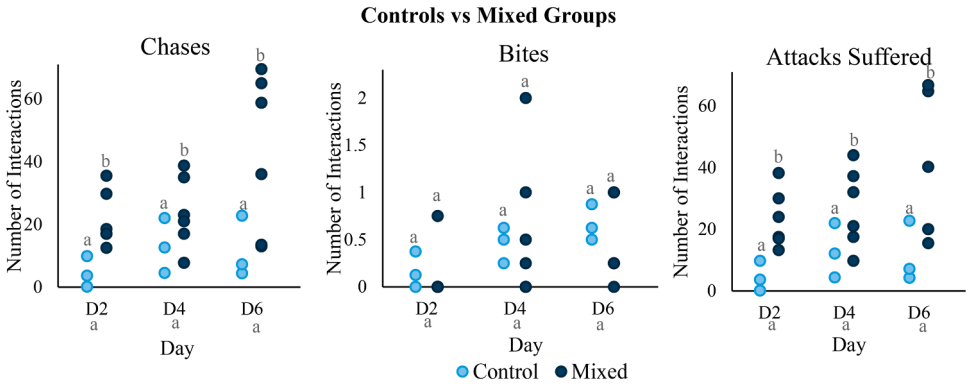

Fig. 1. Conspecific interactions of *S. alburnoides* in the presence of *G. holbrooki* under baseline density. Number of conspecific interactions recorded per *S. alburnoides* individual across three observation days (D2–D6). Light blue markers represent control groups, and dark blue markers represent mixed groups with *G. holbrooki*. Different letters indicate significant differences among days and treatments based on Tukey-adjusted post-hoc comparisons. Panels show: Chases, Bites and Attacks Suffered.

Table 3
Summary of statistical results for behavioral variables of *S. alburnoides* under baseline and high-density conditions. Within mixed groups, behaviors were compared between interaction types (Conspecific vs. Heterospecific), across days (D2, D4, D6), and their interaction (Type × Day). Linear mixed-effects models (LMMs) were applied where assumptions were met; otherwise, non-parametric alternatives (Wilcoxon signed-rank and Friedman tests) were used. Significant results are shown in **bold** ($p < 0.05$). Abbreviations: T= Type effect; D= Day effect; $T \times D$= Type × Day interaction; ns= not significant; —= not applicable/test not performed.

† Total Attacks= Chases + Bites; c = for conspecific interactions; h = for heterospecific interaction

Species	Condition	Behavior	Test	Type (T)	Day (D)	$T \times D$ Interaction
With *G. holbrooki*	Baseline	Chases	Wilcoxon	0.0625	—	—
			Friedman	—	$c = 0.3114$ $h = 0.8465$	—
		Bites	Wilcoxon	0.0975	—	—
			Friedman	—	$c = 0.0617$ h=NA	—
		Escapes	Wilcoxon	0.0625	—	—
			Friedman	—	$c = 0.2001$ $h = 0.7376$	—
		Total Attacks †	Wilcoxon	0.0625	—	—
			Friedman	—	$c = 0.3114$ $h = 0.8465$	—
	High-density	Chases	LMM	***0.0001***	***0.0326***	ns
		Bites	Wilcoxon	0.1814	—	—
			Friedman	—	$c = 0.3679$ h = NA	—
		Escapes	LMM	***0.0004***	0.3790	***0.0208***
		Total Attacks†	LMM	***0.0001***	***0.0347***	ns
With *L. gibbosus*	Baseline	Chases	LMM	***0.0002***	0.1012	ns
		Bites	LMM	***0.0014***	***0.0107***	***0.0168***
		Escapes	LMM	***0.0190***	0.5154	ns
		Total Attacks †	LMM	***0.0001***	0.0905	ns
	High-density	Chases	Wilcoxon	0.1250	—	—
			Friedman	—	$c = 0.1738$ $h = 0.0849$	—
		Bites	Wilcoxon	0.1489	—	—
			Friedman	—	$c = 0.9260$ $h = 0.3679$	—
		Escapes	Wilcoxon	0.1250	—	—
			Friedman	—	$c = 0.1738$ $h = 0.3679$	—
		Total Attacks †	Wilcoxon	0.1250	—	—
			Friedman	—	$c = 0.1738$ $h = 0.1454$	—

When the effect of Day was significant, post hoc pairwise comparisons were conducted using estimated marginal means (emmeans) with Tukey adjustment. Significant differences were detected for *L.* gibbosus bites between D2 and D6 ($p = 0.0220$) and between D4 and D6 ($p = 0.0220$), and for G. holbrooki chases between D2 and D6 ($p = 0.0256$) and total attacks ($p = 0.0273$).

3.2. Higher-density conditions

Under higher-density conditions, behavioral differences between control and mixed groups were less evident. When introduced to *G. holbrooki*, although no significant differences were detected between control and mixed groups for any conspecific behaviors (chases, bites or attacks suffered) (Fig. 6), comparisons within the mixed groups revealed marked differences.

Conspecific chases were more frequent than heterospecific ones ($p < 0.001$), and chase frequency increased over time ($p = 0.0326$), with a

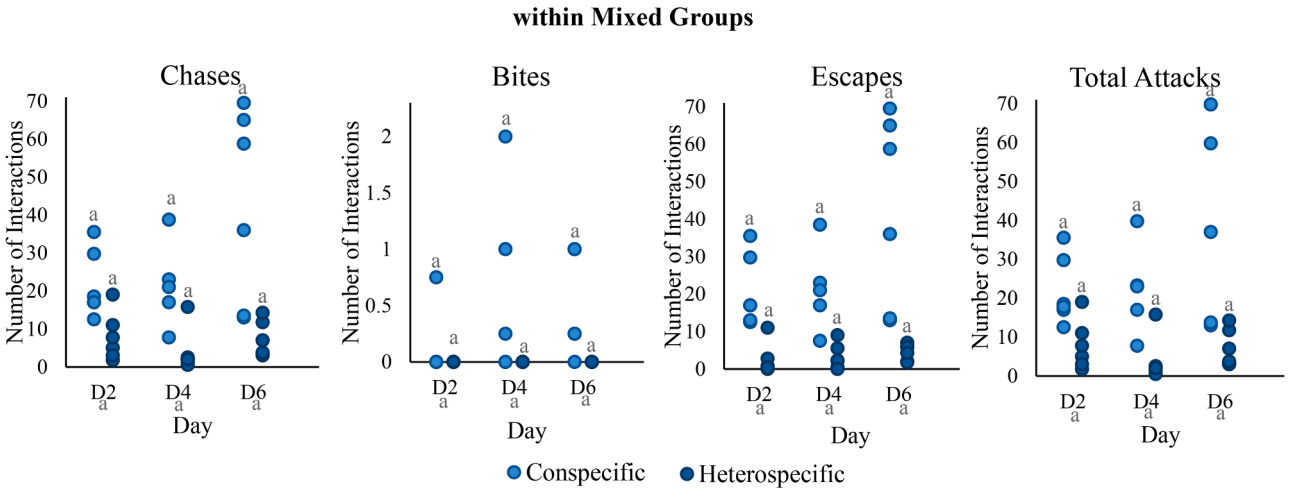

Fig. 2. Conspecific versus heterospecific interactions of *S. alburnoides* with *G. holbrooki* under baseline density. Number of interactions recorded per *S. alburnoides* individual across three observation days (D2–D6). Light blue markers represent conspecific interactions, and dark blue markers represent heterospecific interactions within mixed groups with *G. holbrooki*. Different letters indicate significant differences among days and treatments based on Tukey-adjusted post-hoc comparisons. Panels show: Chases, Bites, Escapes, and Total Attacks.

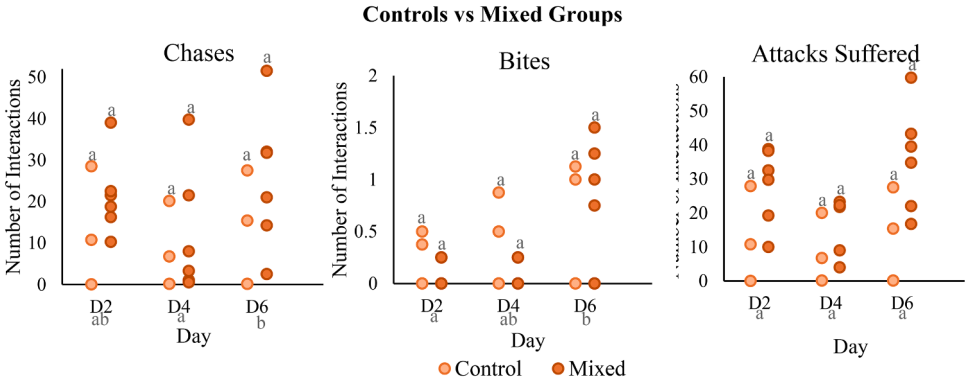

Fig. 3. Conspecific interactions of *S. alburnoides* in the presence of *L. gibbosus* under baseline density. Number of conspecific interactions recorded per *S. alburnoides* individual across three observation days (D2–D6). Light orange markers represent control groups, and dark orange markers represent mixed groups with *L. gibbosus*. Different letters indicate significant differences among days and treatments based on Tukey-adjusted post-hoc comparisons. Panels show: Chases, Bites and Attacks Suffered.

significant difference between day 1 and day 3 ($p = 0.0256$). Conspecific escapes ($p < 0.001$) and total conspecific attacks ($p < 0.001$) were also more frequent than the heterospecific ones (Table 3, Fig. 7). A significant interaction between interaction type and day was found for escapes ($p = 0.0208$).

With *L. gibbosus, S. alburnoides* performed significantly more conspecific chases ($p = 0.021$), bites ($p = 0.0223$) and suffered more attacks ($p = 0.0209$) compared to control groups (Table 2) (Fig. 8).

Within mixed groups exposed to *L. gibbosus*, no significant differences were detected between conspecific and heterospecific behaviors, including chases, bites, escapes, and total attacks (Table 3, Fig. 9). There were no significant differences in the number of attacks suffered and caused during heterospecific interactions for either species ($p > 0.05$).

At the physiological level, both invasive species induced significant reductions in plasma cortisol levels in *S. alburnoides* (exposed to *G. holbrooki*, $p = 0.009$; *L. gibbosus*, $p = 0.02$; Table 4, Fig. 10). In the presence of *G. holbrooki*, there were also increases in forebrain 5-HIAA ($p = 0.021$), and elevated DOPAC/DA ($p = 0.029$) and 5-HIAA/5-HT ratios ($p = 0.029$), suggesting enhanced monoaminergic activity (Table 4, Fig. 6). No significant changes were observed in *S. alburnoides* forebrain monoamines when introduced to *L. gibbosus* individuals, under higher-density conditions.

Exploratory behaviour–physiology correlation analyses are presented in the Supplementary Material (Tables S5–S6; Fig. S11), given the limited sample sizes and the absence of comparable control-group analyses.

4. Discussion

4.1. Lower density conditions

The presence of invasive species markedly altered the behavior of *S. alburnoides*, particularly under baseline (lower) density conditions. From an ecological perspective, increased conspecific aggression in the presence of an invasive species may reflect indirect competition mechanisms rather than direct antagonistic interactions. Invasive fishes can alter perceived resource availability, spatial use, or social stability, leading native individuals to redirect aggression towards conspecifics as they compete for access to food, shelter, or favorable social positions [4,13,47]. Such socially mediated effects of invasions have been reported across freshwater systems, where behavioral disruption often precedes measurable demographic declines [9,24].

When exposed to *G. holbrooki, S. alburnoides* exhibited increased aggression towards conspecifics, as evidenced by a higher frequency of conspecific chases and a greater number of attacks suffered in mixed groups compared to controls. This behavioral escalation suggests that

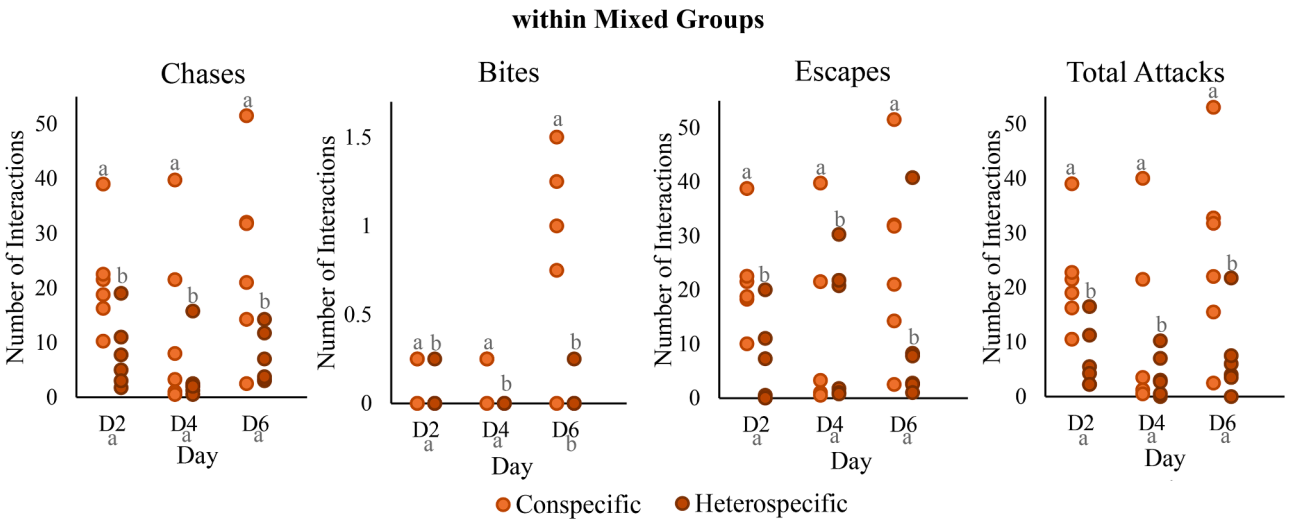

Fig. 4. Conspecific versus heterospecific interactions of *S. alburnoides* with *L. gibbosus* under baseline density. Number of interactions recorded per *S. alburnoides* individual across three observation days (D2–D6). Light orange markers represent conspecific interactions and dark orange markers represent heterospecific interactions within mixed groups with *L. gibbosus*. Different letters indicate significant differences among days and treatments based on Tukey-adjusted post-hoc comparisons. Panels show: Chases, Bites, Escapes, and Total Attacks.

the presence of *G. holbrooki* may act as a social primer, heightening intra-group tension and competition among native individuals. The prevalence of conspecific over heterospecific interactions within mixed groups further indicates that *S. alburnoides* focuses more of its social activity on conspecifics rather than directly confronting the invasive species — a pattern consistent with redirected aggression, often observed in subordinate or socially stressed fish [48,49].

It is important to note that experimental individuals were collected from distinct natural habitats, including both lentic and lotic ecosystems. The differences in habitat structure and natural population densities may have shaped the baseline social experience of individuals prior to laboratory trials. Such background variation is inherent to studies using wild-caught fish and should be considered when interpreting context-dependent behavioral and physiological responses [50,51]. Such background differences may also therefore contribute not only to baseline behavioral tendencies, but also to the distinct ways in which *S. alburnoides* responded to the two invasive species under otherwise similar experimental conditions.

Physiologically, these behavioral shifts were accompanied by a significant decrease in forebrain dopamine (DA) levels in *S. alburnoides* exposed to *G. holbrooki*. However, it is important to note that tissue DA concentrations alone are difficult to interpret, as they reflect the combined pools of intra- and extracellular neurotransmitter and do not directly indicate synaptic release or reuptake dynamics. Although dopaminergic pathways are central to motivation, reward, and social decision-making [52], the absence of significant changes in DOPAC levels or in the DOPAC/DA ratio suggests that increased dopaminergic turnover cannot be conclusively inferred under these conditions. Instead, reduced DA availability may reflect context-dependent modulation of dopaminergic tone associated with altered social processing or motivational state during heightened conspecific aggression, rather than sustained changes in dopaminergic activity per se [51,53,54].

In this case, lower DA levels may indicate increased dopaminergic turnover associated with escalated aggressive behaviors, which is consistent with the behavioral intensification observed. Interestingly, no significant differences were found in cortisol or serotonergic activity under baseline conditions, suggesting that the introduction of *G. holbrooki* was not sufficient to trigger broad HPI-axis changes. Instead, these results may represent an early stage of social tension primarily reflected in behaviour and dopaminergic modulation, without activation of the systemic stress response [51]. Supporting this interpretation, correlation analyses within mixed groups revealed no statistically significant relationships between behavioral and physiological variables. However, non-significant trends — notably a negative association between attacks suffered and cortisol, and a positive one, with both serotonin (5-HT) and its catabolite 5-HIAA — are biologically consistent with HPI-axis suppression under social stress and elevated serotonergic activity in response to repeated social interactions [48,55]. These patterns did not reach statistical significance likely due to small sample size and high interindividual variability, which are known to limit statistical power in behavioral ecology studies [56,57].

In contrast, the presence of *L. gibbosus* did not significantly alter *S. alburnoides* behavior compared to controls, although differences emerged across days in several behaviors. This temporal variation may reflect social habituation or exploratory responses rather than a direct response to this particular invasive species. Within mixed groups however, conspecific interactions were generally more frequent than heterospecific, which again suggests that *S. alburnoides* directs more of its social attention and aggression toward conspecifics, while remaining behaviorally unresponsive to the invasive species [28]. The absence of clear physiological or consistent behavioral disruptions may imply a lower perceived threat from *L. gibbosus* under these conditions.

Importantly, in this case, exploratory correlation analyses (Supplementary Material) suggested positive associations with cortisol, DOPAC, and 5-HIAA levels, suggesting coordinated activation of neuroendocrine and monoaminergic pathways in response to increased social conflict. It is increasingly recognized that monoaminergic activity and HPI-axis output do not necessarily covary in a linear or uniform manner across species or contexts. Several studies have demonstrated dissociations between cortisol dynamics and brain monoamines, with monoaminergic systems responding rapidly to social or environmental challenges, while cortisol responses may be delayed, attenuated, or context dependent [15,16,58]. In social settings, monoamines such as dopamine and serotonin are often involved in modulating motivation, vigilance, and social decision-making, whereas cortisol reflects a broader integrative response to energetic and physiological demands [20,52,54]. The positive association observed here between aggression and both cortisol and monoaminergic markers may therefore reflect a more reactive coping profile under baseline density conditions, in which heightened social interactions translate into increased neuroendocrine engagement [51,59]. Importantly, similar patterns of increased monoaminergic activity occurring independently of, or disproportionate to, cortisol responses

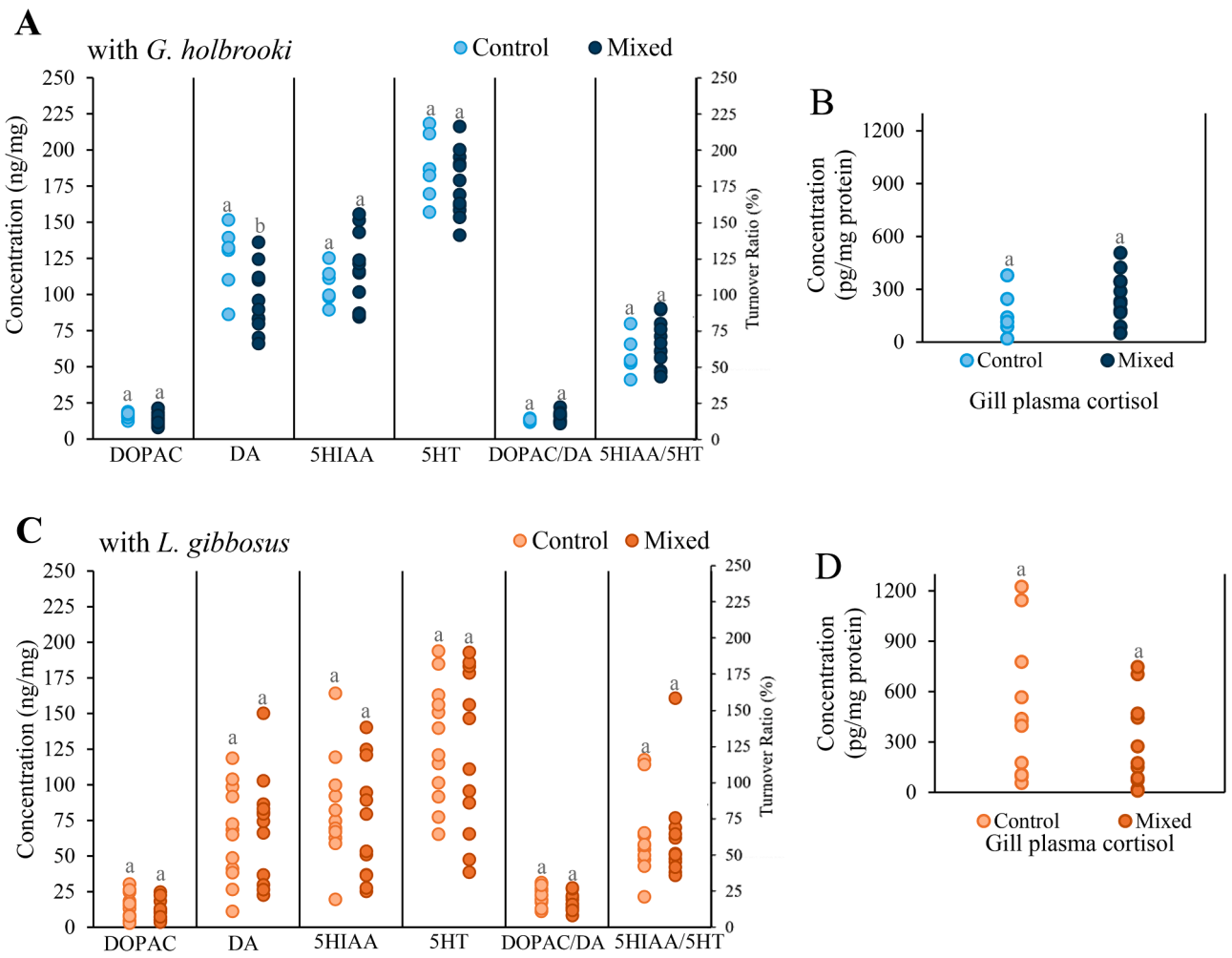

Fig. 5. Physiological responses of *S. alburnoides* to the presence of invasive species under baseline conditions. Concentrations of gill plasma cortisol (pg/mg protein) and forebrain monoamines (ng/g) (dopamine (DA), 3,4-Dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), 5-Hydroxyindoleacetic acid (5-HIAA)) and turnovers ratios (%). Upper panels correspond to groups with *G. holbrooki* (shades of blue), where (A) shows monoamine concentrations and turnover ratios and (**B**) gill plasma cortisol. Lower panels correspond to groups with *L. gibbosus* (shades of orange), where (**C**) shows monoamine concentrations and turnover ratios and (**D**) gill plasma cortisol. Lighter tones represent control groups, darker tones indicate mixed groups. Different letters indicate significant differences among days and treatments based on Tukey-adjusted post-hoc comparisons.

have been reported in other teleost species facing social instability or competition, highlighting the complexity and flexibility of stress regulation in fish [60,61].

Increased conspecific aggression may have important functional consequences. Elevated aggressive interactions are energetically costly and can reduce time allocated to foraging, vigilance, or reproductive behaviors, particularly in small-bodied cyprinids where energy budgets are tightly constrained [13,47,58]. Moreover, heightened intra-group conflict may compromise shoal cohesion, thereby reducing the effectiveness of collective anti-predator strategies and increasing individual predation risk [26].

4.2. Higher-density conditions

Under higher-density conditions, the behavioral response of *S. alburnoides* to invasive species shifted. No significant differences in conspecific behaviors were detected between control and mixed groups with *G. holbrooki,* possibly reflecting a buffering effect of increased group size. Being a schooling species [26], *S. alburnoides* may benefit from the social buffering that larger group sizes can provide, reducing the perceived need to adjust behavior immediately in response to non-native species [61]. Nevertheless, comparisons within mixed groups revealed consistent patterns: conspecific chases, escapes, and total attacks were all more frequent than heterospecific ones, and the frequency of chases increased significantly across days. This escalation suggests that prolonged exposure to *G. holbrooki*, even in larger groups, may lead to cumulative social conflict. Neurochemically, this was paralleled by increased serotonergic catabolite (5-HIAA), higher turnover ratios (5-HIAA/5-HT and DOPAC/DA), and a marked reduction in plasma cortisol levels. Such changes are consistent with established social conflict, where monoaminergic systems are mobilized to regulate persistent vigilance and aggression, while HPI activity becomes down-regulated [54,55]. However, the simultaneous increase in serotonergic turnover and reduction of circulating cortisol may also reflect the action of negative feedback mechanisms that limit HPI-axis activation once the stressor becomes predictable or long term stabilized. As discussed by Bernier *et al.* [62], efficient glucocorticoid feedback can suppress cortisol synthesis through feedback inhibition at both the pituitary and hypothalamic levels, thereby preventing excessive or prolonged endocrine activation. Although this hypothesis cannot be directly verified here, given that cortisol receptor activity was not assessed, it offers a plausible explanation for the observed attenuation of cortisol despite elevated serotonergic activity. Although no significant correlations were found, the negative trend between attacks suffered and cortisol reveals

Table 4
Results of statistical comparisons for gill plasma cortisol levels and brain monoamines in *S. alburnoides* under baseline and high-density conditions. Comparisons were made between control and mixed groups exposed to either *G. holbrooki* or *L. gibbosus*. Variables include gill plasma cortisol, brain monoamine concentrations (dopamine (DA), 3,4-Dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), 5-Hydroxyindoleacetic acid (5-HIAA)), and their respective turnover ratios (DOPAC/DA and 5-HIAA/5-HT). Statistical tests used include ANOVA, Welch's ANOVA, and Kruskal-Wallis, as appropriate. Significant results are indicated in **bold** ($p < 0.05$).

Species	Condition	Compound	Test	*p*-value
With *G. holbrooki*	Baseline	Gill Plasma Cortisol	ANOVA	0.1460
		DOPAC	ANOVA	0.5040
		DA	ANOVA	***0.0224***
		5HIAA	ANOVA	0.4970
		5HT	ANOVA	0.3390
		DOPAC/DA ratio	Welch's ANOVA	0.0749
		5HIAA/5HT ratio	Welch's ANOVA	0.3350
	High-density	Gill Plasma Cortisol	Kruskal-Wallis	***0.0093***
		DOPAC	ANOVA	0.4060
		DA	ANOVA	0.9930
		5HIAA	ANOVA	***0.0208***
		5HT	ANOVA	0.9540
		DOPAC/DA ratio	Kruskal-Wallis	***0.0288***
		5HIAA/5HT ratio	Kruskal-Wallis	***0.0288***
With *L. gibbosus*	Baseline	Gill Plasma Cortisol	ANOVA	0.2430
		DOPAC	ANOVA	0.3810
		DA	ANOVA	0.7560
		5HIAA	ANOVA	0.5700
		5HT	ANOVA	0.7720
		DOPAC/DA ratio	ANOVA	0.0697
		5HIAA/5HT ratio	Kruskal-Wallis	0.7119
	High-density	Gill Plasma Cortisol	Welch's ANOVA	***0.0189***
		DOPAC	Kruskal-Wallis	0.8802
		DA	ANOVA	0.6640
		5HIAA	Kruskal-Wallis	0.2744
		5HT	Kruskal-Wallis	0.4510
		DOPAC/DA ratio	Kruskal-Wallis	0.7345
		5HIAA/5HT ratio	Kruskal-Wallis	0.7919

the pattern of HPI-axis suppression under sustained social conflict [51,55], despite the limitations imposed by small sample size.

Interpretation of these density-dependent patterns should also consider the potential influence of prior social experience in the wild. Although fish were collected from sites where exposure to invasive species was expected to be limited, natural population densities at the sampling locations were not quantified, and therefore baseline exposure to crowding, competition, and social instability may have differed among individuals before experimental manipulation. In addition, although catch per unit effort (CPUE) is often used as a proxy for relative abundance and social density in wild fish populations, such data were not available for the sampling sites used in this study [63]. Consequently, potential links between natural population density, prior social experience, and individual variation in behavioral or physiological traits could not be directly assessed. This limitation is inherent to studies relying on wild-caught individuals and should be considered when interpreting density-dependent responses observed under laboratory conditions.

Despite the increase in aggressive interactions and monoaminergic activity under high-density conditions, cortisol levels measured in the gills of *S. alburnoides* were reduced. It is important to note that branchial cortisol does not necessarily reflect circulating plasma concentrations, but rather integrates multiple processes, including local uptake, diffusion from water, metabolic clearance, and tissue-specific regulation [60,64]. Consequently, changes in branchial cortisol should be interpreted cautiously and not assumed to represent a direct proxy of systemic HPI-axis activation.

In the present study, cortisol was quantified exclusively in gill tissue, a matrix that has been proposed as a non-invasive indicator of stress in teleosts but remains comparatively uncommon and species-dependent in its interpretation. Previous validation of this approach has been conducted primarily in model species such as rainbow trout and zebrafish [17], which exhibit markedly different physiological responses and baseline cortisol dynamics. As no concurrent measurements of plasma or whole-body cortisol were performed, and baseline gill cortisol levels have not previously been reported for *S. alburnoides*, it is not possible to infer the absolute magnitude of the stress response or directly compare these values with those obtained in other species. Consequently, comparisons with acute stress responses in zebrafish should not be regarded as a validation of the methodology, but rather as contextual reference points.

Importantly, branchial cortisol reflects tissue-specific dynamics influenced by multiple processes, including diffusion, local metabolism, and clearance, and should therefore be interpreted cautiously and not as a direct proxy for systemic HPI-axis activation. Within this framework, the reduction in gill cortisol observed under high-density conditions may reflect adaptive modulation of cortisol regulation in response to current social challenges. While chronic stress has been shown in several teleost species to induce attenuation or downregulation of cortisol responses through habituation or enhanced negative feedback (e.g. [51,55,62]), other studies report prolonged elevation of cortisol under chronic or unpredictable stressors (e.g. [15,16]). This variability highlights that cortisol dynamics are context-, species-, and tissue-dependent. In this study, the observed pattern of reduced branchial cortisol, together with increased aggression and elevated monoaminergic turnover, suggests a context-regulated physiological state and not an absence of social challenge.

When exposed to *L. gibbosus* under higher-density conditions, *S. alburnoides* exhibited pronounced behavioral changes. In mixed groups, conspecific chases, bites, and attacks suffered were significantly higher than in control groups. However, comparisons within mixed groups revealed no significant differences between conspecific and heterospecific interactions across behavioral categories (chases, bites, escapes, and total attacks). These results indicate that, although the presence of *L. gibbosus* was associated with increased intra-specific aggression amongst natives under crowded conditions, there is no statistical support for differential targeting of conspecifics versus heterospecifics. The absence of significant physiological correlations in this condition suggests a disconnect between behavioral and physiological reactivity, potentially due to ceiling effects in social buffering or altered perception of risk under crowding.

Alterations in monoaminergic activity and cortisol regulation have consequences that extend beyond behavioral outputs. Persistent modulation of these pathways can influence individual coping styles, behavioral flexibility, and long-term fitness, potentially reshaping social structure and population resilience under invasion pressure [52,59]. At the population level, such neuroendocrine shifts may translate into reduced cooperative behaviors and increased vulnerability to additional stressors, particularly in systems already affected by habitat fragmentation and environmental change [4,24].

Although fish were collected from locations where naïve individuals were expected, no quantitative estimates of natural population density were available. Differences in baseline social environment may therefore influence behavioral tendencies and stress physiology expressed under experimental conditions [13,47,65]. Variation in prior social

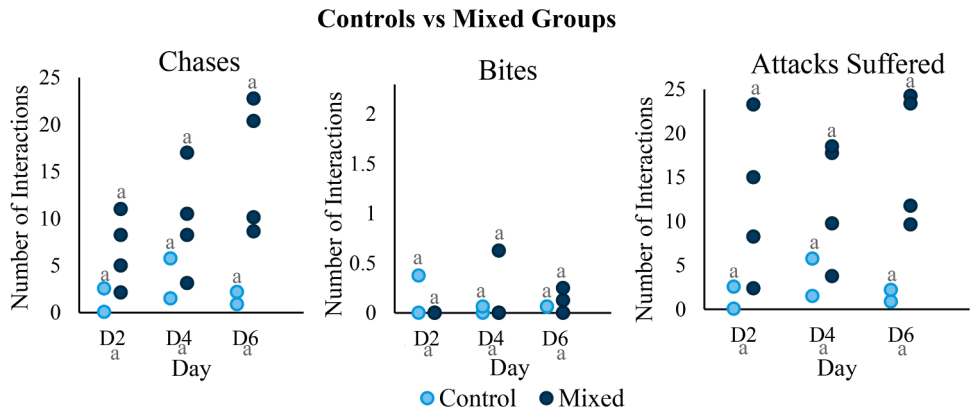

Fig. 6. Conspecific interactions of *S. alburnoides* in the presence of *G. holbrooki* under high-density conditions. Number of conspecific interactions recorded per *S. alburnoides* individual across three observation days (D2–D6). Light blue markers represent control groups, and dark blue markers represent mixed groups with *G. holbrooki*. Different letters indicate significant differences among days and treatments based on Tukey-adjusted post-hoc comparisons. Panels show: Chases, Bites and Attacks Suffered.

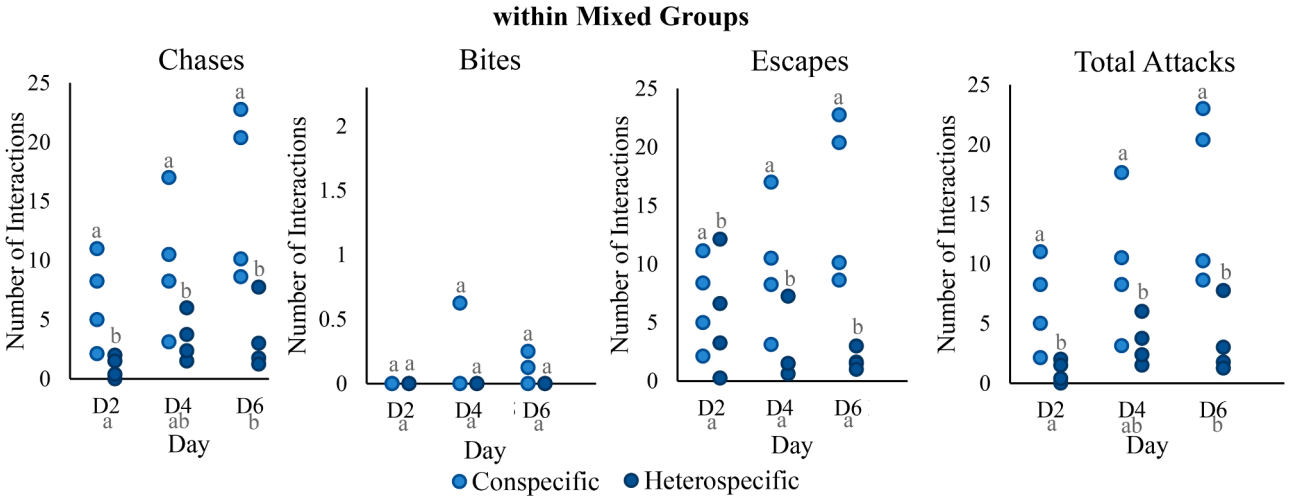

Fig. 7. Conspecific versus heterospecific interactions of *S. alburnoides* with *G. holbrooki* under high-density conditions. Number of interactions recorded per *S. alburnoides* individual across three observation days (D2–D6). Light blue markers represent conspecific interactions, and dark blue markers represent heterospecific interactions within mixed groups with *G. holbrooki*. Different letters indicate significant differences among days and treatments based on Tukey-adjusted post-hoc comparisons. Panels show: Chases, Bites, Escapes, and Total Attacks.

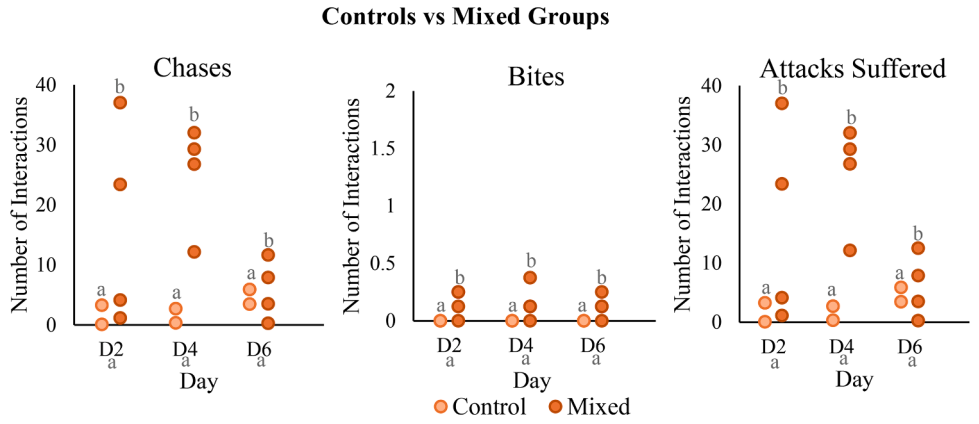

Fig. 8. Conspecific interactions of *S. alburnoides* in the presence of *L. gibbosus* under high-density conditions. Number of conspecific interactions recorded per *S. alburnoides* individual across three observation days (D2–D6). Light orange markers represent control groups, and dark orange markers represent mixed groups with *L. gibbosus*. Different letters indicate significant differences among days and treatments based on Tukey-adjusted post-hoc comparisons. Panels show: Chases, Bites and Attacks Suffered.

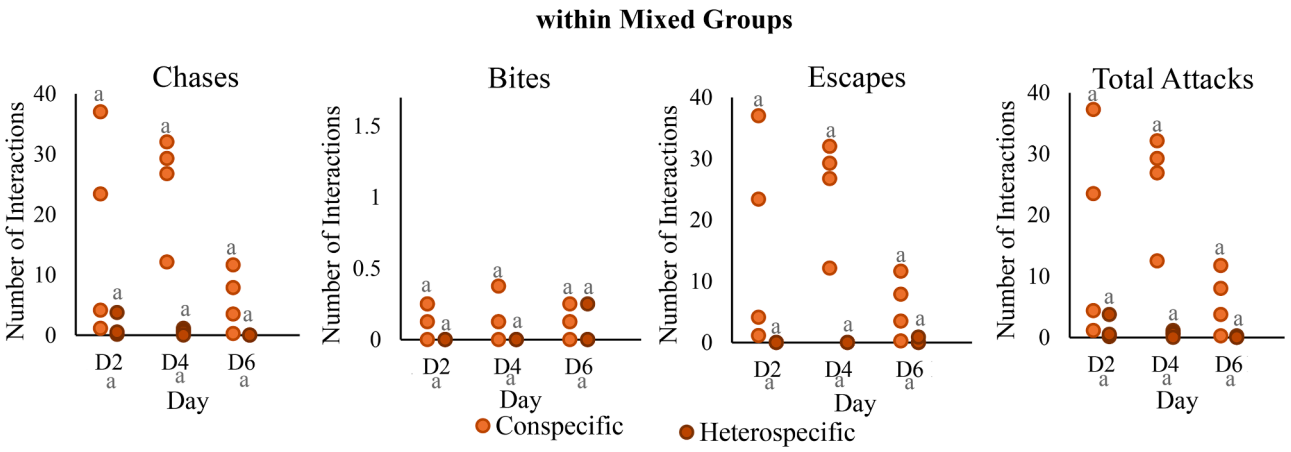

Fig. 9. Conspecific versus heterospecific interactions of *S. alburnoides* with *L. gibbosus* under high-density conditions. Number of interactions recorded per *S. alburnoides* individual across three observation days (D2–D6). Light orange markers represent conspecific interactions and dark orange markers represent heterospecific interactions within mixed groups with *L. gibbosus*. Different letters indicate significant differences among days and treatments based on Tukey-adjusted post-hoc comparisons. Panels show: Chases, Bites, Escapes, and Total Attacks.

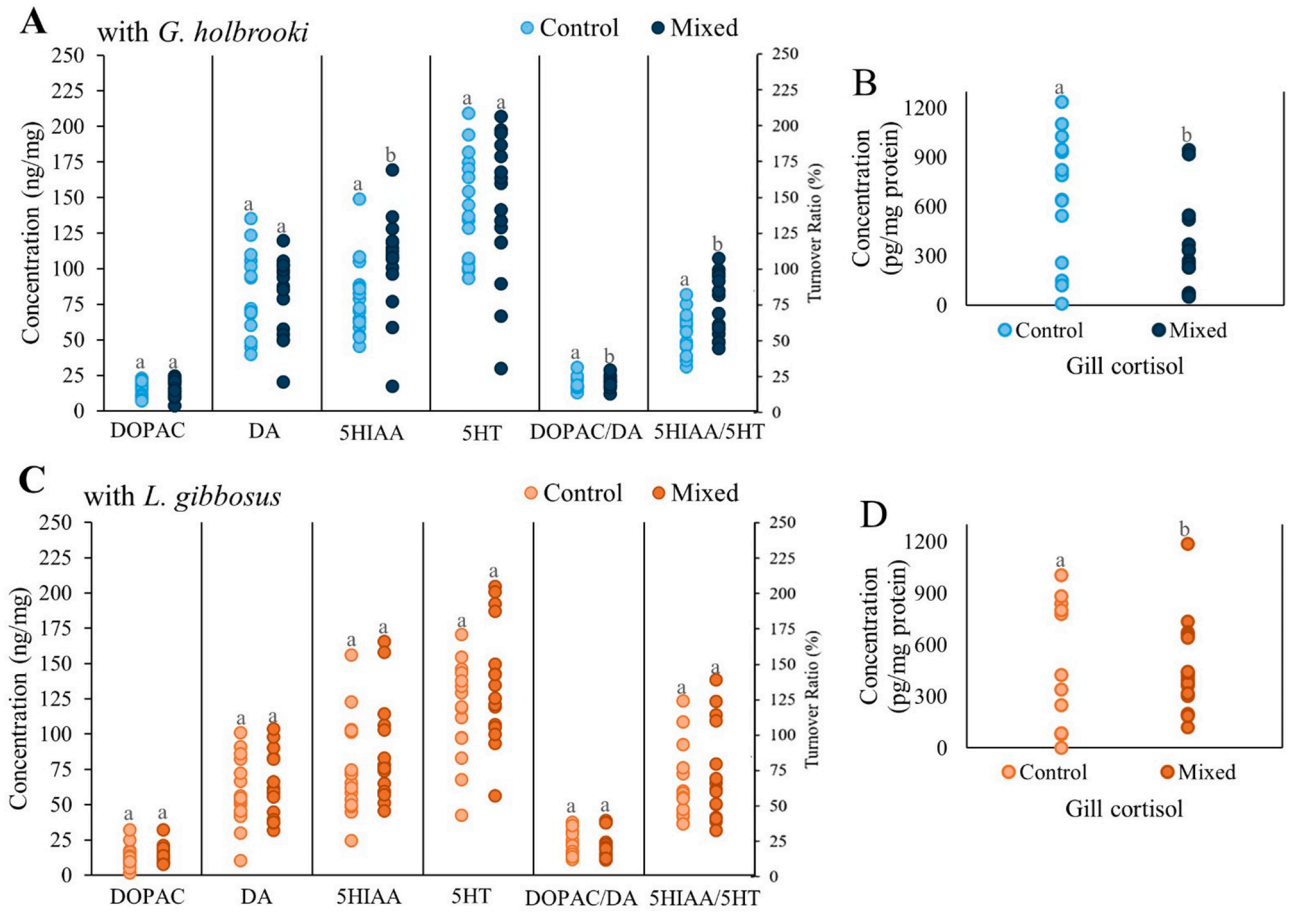

Fig. 10. Physiological responses of *S. alburnoides* to the presence of invasive species under high-density conditions. Concentrations of gill plasma cortisol (pg/mg protein) and forebrain monoamines (ng/g) (dopamine (DA), 3,4-Dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), 5-Hydroxyindoleacetic acid (5-HIAA)) and turnovers ratios (%). Upper panels correspond to groups with *G. holbrooki* (shades of blue), where (**A**) shows monoamine concentrations and turnover ratios and (**B**) gill plasma cortisol. Lower panels correspond to groups with *L. gibbosus* (shades of orange), where (**C**) shows monoamine concentrations and turnover ratios and (**D**) gill plasma cortisol. Lighter tones represent control groups, darker tones indicate mixed groups. Different letters indicate significant differences among days and treatments based on Tukey-adjusted post-hoc comparisons.

experience could help explain why *G. holbrooki* and *L. gibbosus* elicited distinct behavioral and physiological profiles to *S. alburnoides*, as well as why some effects became more pronounced under higher-density conditions. Individuals originating from more stable social environments may respond more strongly to experimentally imposed crowding or novel social challenges, whereas those from naturally dense or

fluctuating systems may exhibit greater behavioral tolerance or altered neuroendocrine regulation [14,51].

These considerations are particularly relevant when interpreting the apparent buffering effects observed under higher-density conditions. While increased group size can reduce perceived risk through social buffering, its effectiveness likely depends on previous experience with crowding and social instability. Thus, background differences in natural population structure may modulate the balance between behavioral adjustment and physiological regulation, influencing whether increased density leads to stress attenuation or escalation [55,61].

Although these factors could not be directly controlled in the present study, explicitly acknowledging their potential influence strengthens the ecological interpretation of the results. Importantly, the consistent behavioral patterns observed across replicates and the convergence between behavioral and neuroendocrine indicators suggest that the main conclusions remain robust, while highlighting avenues for future research integrating field-based population data with controlled experimental approaches.

A limitation of this study is that behavioral and physiological correlations were calculated at the tank level rather than for individual fish. This approach inevitably obscures within-group variability in social dynamics, such as differences between dominant and subordinate individuals, which are highly relevant for interpreting stress-related variables [49,65]. As a result, it is possible that individuals contributing most to aggressive outputs differ in their physiological responses from those receiving aggression, a nuance that cannot be captured when data are pooled at the group level. Moreover, the use of wild-caught individuals in experiments addressing social interactions and density is methodologically challenging and can introduce uncontrolled variation due to differences in prior social experience, habitat conditions, and population structure.

Physiological parameters also varied among sampling periods, reflecting seasonal and temporal variability inherent to studies conducted with wild-caught individuals across extended timeframes. Because sampling periods were partially associated with experimental blocks, these effects could not be fully disentangled from treatment effects and should therefore be interpreted cautiously.

Despite these limitations, this study provides an integrative framework linking behavioral and neuroendocrine responses of a native freshwater fish to the presence and density of invasive species. By combining behavioral and physiological endpoints, our findings highlight the capacity of *S. alburnoides* to adjust to complex social and ecological stressors, while also revealing potential vulnerabilities under invasion pressure. Understanding these mechanisms is crucial for predicting how native species will cope with ongoing biological invasions, particularly as environmental change and anthropogenic stressors continue to intensify. Studies such as this one are essential to inform conservation strategies in Mediterranean freshwater systems, where endemic species already face cumulative threats from habitat alteration, climate change, and invasive fauna.

The experimental design focused on comparing conspecific groups of *S. alburnoides* with mixed groups containing invasive species. Although the inclusion of an additional native heterospecific could have helped disentangle responses to heterospecific presence per se from responses specifically elicited by invasive species, this approach was not adopted due to logistical and ethical constraints. Several native freshwater species of potential interest are protected or of conservation concern and obtaining authorization to experimentally manipulate multiple at-risk species was not feasible within the scope of this study.

Group sizes were selected based on previous observations of normal shoaling behavior in *S. alburnoides*, with four individuals representing the minimum number at which stable group behavior could be reliably observed, while also minimizing the number of wild-caught individuals required. Alternative designs, such as territorial "resident–intruder" paradigms in which native fish establish prior residency before the introduction of competitors, could provide additional ecological realism. However, such approaches address different ecological questions related to territoriality rather than the density- and composition-driven social dynamics that were the focus of the present study. These alternatives represent valuable directions for future research.

4.3. Concluding remarks

Taken together, our findings show that the two invasive species affect *S. alburnoides* in distinct but complementary ways. While exposure to *G. holbrooki* was more consistently associated with neuroendocrine modulation and cortisol attenuation under crowding, *L. gibbosus* primarily intensified intra-specific aggression among native fish, particularly at higher densities. These divergent effects highlight that biological invasions can alter both social dynamics and physiological regulation, but the pathways may differ between invaders. Moreover, the putative change in social dynamics may reduce overall fitness and impair cooperative or affiliative behavior necessary for group cohesion, especially under conditions of environmental stress or resource limitation [47,58]. In higher-density conditions — a common consequence of seasonal droughts and habitat fragmentation in Mediterranean rivers [10,32] — these effects may be exacerbated. As water levels drop and fish aggregate in small, isolated pools, the probability of intense intra- and interspecific interactions also increase [8]. While *S. alburnoides* may initially benefit from shoaling in such scenarios, prolonged exposure to invasive species may lead to measurable increases in conspecific aggression and altered neurophysiology. Such chronic conflict conditions may compromise energy budgets, reproductive behavior, or predator awareness, ultimately affecting survival and population stability [4,11]. In this light, biological invasions must be considered not only as ecological disturbances but also as drivers of behavioral and physiological change, reshaping social dynamics in native communities. As climate change increases the frequency of drought and habitat fragmentation in Mediterranean systems, the compounded impact of crowding and biological invasions may threaten the resilience of native populations. Understanding the interplay between behavioral strategies and physiological regulation is therefore essential for anticipating population stability and informing conservation priorities in freshwater ecosystems.

Data availability statement

All data supporting the findings of this study are available from the corresponding author upon reasonable request. Processed datasets and statistical scripts can be made available through an open repository upon acceptance for publication.

CRediT authorship contribution statement

Marta Luiz: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Filipe Banha:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Gonçalo Brito:** Methodology, Investigation. **Bianca Cambiaghi e Silva:** Writing – review & editing, Formal analysis. **Pedro Anastácio:** Writing – review & editing, Resources, Funding acquisition. **Manuel Gesto:** Writing – review & editing, Formal analysis, Data curation. **Marta C. Soares:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest related to this work.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2026.115298.

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