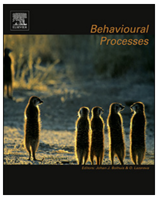
Behavioural Processes 157 (2018) 495–501

Contents lists available at ScienceDirect

Behavioural Processes

journal homepage: www.elsevier.com/locate/behavproc



Shoal sex composition and predation risk influence sub-adult threespine stickleback shoaling decisions

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ARTICLE INFO

Keywords:
Fish
Gasterosteus aculeatus
Group formation
Sexual segregation
Sexually monochromatic stage

ABSTRACT

Group living reduces individual predation risk most effectively when group members are behaviorally and phenotypically similar. Group preferences are influenced by the individual, the members of the shoal, and the environmental conditions. While shoaling behavior has been studied extensively in the threespine stickleback (*Gasterosteus aculeatus*), it is unclear whether the sex of shoal mates influences the shoal preference of non-reproductive males and females and how this changes under increasing predation risk. Although non-reproductively active sticklebacks are sexually monochromatic in appearance, sex-related differences may result in sexual segregation when shoaling. Here we show that male and female sub-adult threespine sticklebacks had contrasting preferences for shoal mate sex, and that this preference was dependent on the level of predation risk during standardized experimental choice tests. In detail, test fish shoal with the opposite sex within low predation risk trials and with same-sex shoals within high predation risk trials. This difference might be linked to activity patterns; test males were more active than females. Our results demonstrate that differences between the sexes in a species with a sexually monochromatic non-reproductive stage can result in sex-related shoaling preferences. Most studies examining sexual segregation focus on sexually dimorphic species, but these results highlight the potentially widespread occurrence of sexual segregation beyond the sexually dimorphic reproductive stage.

1. Introduction

Living in groups comes with many advantages, such as an increase in food acquisition, reduction in predation risk, and energy-efficient travel (Krause and Ruxton, 2002). Forming groups minimizes predation risk when all individuals are phenotypically and behaviorally similar. Referred to as the confusion and oddity effects, groups comprised of similar individuals overwhelm predators; the sheer number of potential prey items confuses the predator and the similarity of all individuals hinders the predator's ability to focus on any one prey item (Landeau and Terborgh, 1986; Theodorakis, 1989). As with any behavior, there are costs and benefits to balance when forming groups. To increase the benefits of group formation, individuals prefer to associate with individuals which resemble themselves in appearance and behavior, such as individuals of the same species (Krause et al., 1996), similar body size (Hoare et al., 2000), and similar coloration and pattern (McRobert and Bradner, 1998). However, the benefits of living in groups are offset by costs such as increased parasite transmission (Richards et al., 2010; Johnson et al., 2011), competition for food (Ranta and Lindström, 1990), and aggressive interactions among conspecifics (Krause and Ruxton, 2002). Balancing these advantages and disadvantages results in different grouping behavior under different conditions.

When sexually dimorphic, males and females might have different energy requirements and varying risks of predation, resulting in contrasting space use and behavior (Ruckstuhl and Clutton-Brock, 2005). As group formation often acts to reduce an individual's risk of predation, differing predation risk for males and females would lead to differences in grouping behavior, potentially even sexual segregation, which is defined as non-random distribution of males and females (Ruckstuhl and Neuhaus, 2002). Sexual segregation is widespread in social ungulate species, where there is a distinct sexual dimorphism (Ruckstuhl, 2007). It has been previously shown in the sexually dimorphic reproductive guppy (*Poecilia reticulata*) that males and females exhibit distinct behaviors when shoaling and choose shoals based on different characteristics, resulting in sexual segregation under predation risk (Griffiths and Magurran, 1997; Magurran, 1998; Kelley et al., 1999; Croft et al., 2004; Richards et al., 2010; Barbosa et al., 2016). Within-group sexual segregation was found in a slightly dimorphic

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https://doi.org/10.1016/j.beproc.2018.06.009
Received 11 October 2017; Received in revised form 8 June 2018; Accepted 18 June 2018

species of whirligig beetles (*Dineutes discolor*) immediately after a simulated predator attack, suggesting that positioning of the sexes is dependent on predation susceptibility (Romey and Galbraith, 2008). Furthermore, in zebrafish (*Danio rerio*), both males and females prefer to shoal with females, possibly as males may be more interested in mating and females more interested in avoiding male harassment (Ruhl and McRobert, 2005; Etinger et al., 2009; Snekser et al., 2010). Although monomorphic males and females do not differ in appearance, their behavior may be profoundly different due to differing activity levels, energy needs, and reproductive investments, resulting in sex differences in predation risk and shoal preference and sexual segregation mediated by social factors (social segregation; Ruckstuhl, 2007; see also Wearmouth and Sims, 2008). In brown-headed cowbirds (*Molothrus ater*), association preferences in both sexually monomorphic juvenile and dimorphic adult flocks are observed, with females preferring females (Kohn et al., 2011). In the sexually monomorphic European minnow (*Phoxinus phoxinus*), Griffiths et al. (2014) found that non-reproductively active males preferentially shoal with males while females show no preference and that males are more active than females. However, to the best of our knowledge there are no other studies examining sex differences in shoaling behavior in monomorphic non-reproductive stages of species. Sex-related shoaling differences are clearly applicable to species with a monomorphic, or more specifically monochromatic, non-reproductive stage and should be more extensively studied.

The threespine stickleback (*Gasterosteus aculeatus*) is a model organism for behavioral studies, including shoaling (reviewed in Huntingford and Ruiz-Gomez, 2009). This species has a large sexual dichromatism; males have blue eyes and red throats, but adult sticklebacks are monochromatic outside of the breeding season and while juvenile or sub-adult (Bell and Foster, 1994). Many studies on the shoaling behavior of sticklebacks have used non-reproductively active individuals but have ignored the possible influence of sex (e.g. Ranta and Lindström, 1990; Peuhkuri, 1998; Barber, 2003; Frommen and Bakker, 2004; Modarressie et al., 2006; Frommen et al., 2007; Harcourt et al., 2009; but see Ward et al., 2004). The prominent sexual dichromatism and difference in body shape and size exhibited by sticklebacks during the breeding season likely alters the behavior, activity, and predation risk of males and females throughout their lifetime. Sex-related behavioral differences such as boldness are also present in non-reproductively active fish; males are more bold and exploratory (King et al., 2013; Mamuneas et al., 2015; Velando et al., 2017). Furthermore, males and females differ in parasite load (Arnold et al., 2003), migratory behavior (Cano et al., 2008), feeding mechanism (McGee and Wainwright, 2013), and gene expression (Velando et al., 2017). These sex-related differences, such as behavior, may lead to distinct anti-predator behavior when shoaling, which may change depending on predation risk.

We sought to investigate the shoaling behavior and preference of sub-adult threespine sticklebacks with respect to sex and predation risk. By presenting a stickleback of a known sex with an entirely male and entirely female size-matched shoal (each consisting of four individuals) and allowing for visual and olfactory cue transmission, we quantified the association time with each sex to determine shoaling preferences under low predation risk (novel environment, tap water) and high predation risk (novel environment plus perch water (*Perca fluviatilis*)). We hypothesized that (i) males and females have different shoaling preferences and behave differently when shoaling under low or high predation risk, with males being more active due to their more bold personality (e.g. King et al., 2013) and (ii) that both sexes will shoal more when the threat of predation is high. Furthermore, we predicted that sticklebacks assort by sex which might increase survival because of the enhanced confusion effect if males and females have a different predation risk and activity rate due to their behavioral differences.

2. Material and methods

2.1. Experimental subjects and housing conditions

The threespine sticklebacks used in this study were from an F1 generation from a large anadromous population from the island of Texel in the Netherlands. Parental fish were collected in March 2015 during the spring migration into rivers and streams. The F1 generation comprised 32 families (approximately 1000–1200 individuals) and was bred in May and June of 2015, each parent used only once. The juveniles were raised in the laboratory under standardized conditions and kept in a large outdoor tank (750 L, flow rate 3 L/min) for approximately 4-5 months prior to the beginning of the study. For the experiments, individuals with no visible sexual dichromatism were randomly selected from the outdoor tank by catching a group with a net. The standard length of fish from this group was measured and individuals between 4.0 and 4.5 cm were selected to size-match fish, and the tip of the first spine was clipped off and used in molecular analysis for sex identification (Bakker et al., 2017). During sexing, individuals were kept in separate small plastic tanks which contained 1 L of aged tap water and an air stone. After sex identification, sticklebacks were randomly assigned to the shoal-fish tank or test-fish tank for their sex (see below). Due to the large amount of F1 individuals and randomization of the selection of test and shoal individuals, any effect of familiarity or kinship on shoaling decisions (e.g. Frommen et al., 2007) during the experiment should be negligible.

The perch, whose scent functions as predator cue for sticklebacks (Frommen et al., 2011), originated from the floodplain of the Rhine River. Individuals were housed together in a large outdoor tank (750 L, flow rate 3 L/min) prior to the experiment.

All experimental fish were kept in an air conditioned room (17 °C ± 1 °C) under winter conditions (8L:16D). Illumination was provided by natural daylight fluorescent lamps (Truelight T8/18 W, T8/36 W, T8/58 W, with a UV-blocking tube guard). Identical tanks measuring 45 cm × 30 cm × 30 cm, filled to 25 cm with aged tap water, and equipped with two air stones housed shoal fish, test fish, and individual perch. All tanks were visually isolated from one another using opaque grey plastic. A maximum of 16 sticklebacks were kept in each shoal-fish or test-fish tank, with separate tanks for each sex. Fish were not kept in the test-fish tank longer than three days; if a fish had not been tested after three days it was relocated to the shoal-fish tank to exclude potential habituation effects. During the experiment, six perch were kept in separate tanks, which additionally had a water filter containing filter wool and two clay flower pots. All perch tanks were covered to prevent escape. For the low predation risk trials, aged tap water was kept in two large tanks that were refilled at the end of each day.

Stickleback and perch were fed with defrosted *Chironomus* larvae *ad libitum* in the afternoon daily when experiments were finished. After at least 15 min for feeding, the excess food and fish excretions were removed and water refilled. Sticklebacks pending DNA analysis in fauna boxes were not fed. Fish were not used in trials if they had not been fed the day before.

2.2. Experimental design

Experimental trials ran Monday through Friday from February 29th through March 11th, 2016, between 10:00 and 17:00. The sequence of predation risk level, sex of test fish, and whether the shoal on the left was male or female were distributed randomly throughout each day. A total of 40 usable trials were conducted, 10 for each low or high predation risk with a male or female test fish. Two other trials were excluded, due to a lethargic shoal fish and a measuring error of a shoal fish.

The experimental tank (70 cm × 35 cm × 35 cm) was split into three sections and surrounded by an opaque lining (Fig. 1). The sections

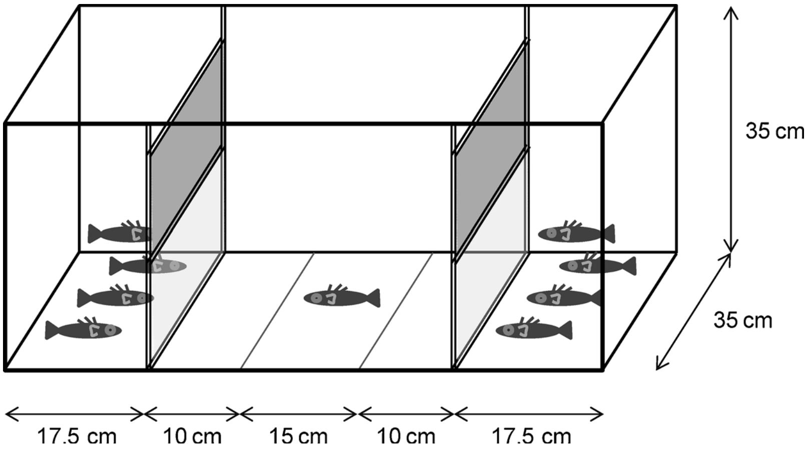

Fig. 1. Experimental tank used for the shoal choice experiment. During trials the opaque partitions were raised, and sections were separated by perforated transparent partitions (height of both partitions is 17 cm). Test fish were placed in the central section; female and male shoals composed of 4 individuals were placed in opposite peripheral sections. Lines indicating preference zones in the central section were drawn on the bottom of the tank with permanent marker.

were separated by a removable opaque partition and a stationary perforated transparent partition allowing both visual and olfactory cues to be transmitted. The shoals, consisting of four individuals, were placed in the peripheral sections, and the test fish was placed in the central section. There were two 10 cm preference zones within the central section directly adjacent to each peripheral shoaling section (Fig. 1). Natural daylight fluorescent lamps (Truelight T8/18 W, T8/36 W, T8/58 W, with a UV-blocking tube guard) hung above the experimental tank, and a Logitech QuickCam® Pro 9000 webcam was attached to the center of the light for video recording. A black curtain surrounded the experimental tank to reduce disturbance.

For each trial, the experimental tank was filled with water to a depth of 10 cm (24 L), either aged tap water for the low predation trials or an equal amount of water from each of the six perch tanks for the high predation trials. Although the low predation trials included no predator cues, it was likely perceived as a risky situation as the experimental tank was a novel environment and the test individual had undergone a short period of isolation. Four fish of each sex, randomly removed from the shoal-fish tanks, were placed in the peripheral shoal sections, the test fish was placed in the middle of the central section, and the curtain was closed. After five minutes of acclimation, the opaque partitions were removed externally with a pulley system to avoid disturbance. The trial began once the test fish had entered the second preference zone and lasted 10 min. At the end of each trial, the mass (mg) and standard length (mm) of all fish were recorded. Condition index was calculated as 100 × mass / standard length^3 after Bolger and Connolly (1989). Relative condition index was calculated as (test fish condition index – mean condition index of shoal fish) / (test fish condition index + mean condition index of shoal fish). The experimental tank and partitions were cleaned using 3% H_2O_2 to remove all odors. Shoal fish were kept in the laboratory for the duration of the experiment. In order to avoid pseudoreplication, test fish were used for only one trial before being returned to the outdoor holding tank, where they were easily identifiable by their clipped dorsal spine. Shoal fish were used for more than one trial within each day, but individuals were exchanged to ensure that each shoal was composed of different individuals.

2.3. Data analysis

Videos were recorded using Windows Media Encoder (version 9.0). Trials were watched on VLC media player (version 1.1.8), and the observer was blind to the location of the male and female shoals and predation treatment. For each trial, the time the test fish spent in each of the two preference zones and the total number of visits to each preference zone were determined. The test fish was considered in a preference zone when the pectoral fins were within the zone, and all preference zone visits were counted regardless of the duration and the time elapsed between visits.

Statistical analyses were done using R 3.0.2. (R Development Core Team, 2013). Independent samples t-tests, paired t-tests, and two-sample Wilcoxon tests (when data significantly deviated from normal distribution) were used to determine significance of sex differences in body length, body condition, and shoaling activity.

To examine the influence of test fish sex, shoal fish sex, and predation risk on shoaling preferences, linear mixed effect models (lme) were fitted using the lme function in the nlme library (Pinheiro et al., 2016). In each model the absolute time of shoaling with each shoal was used as the dependent variable, and trial number was included as a random factor to control for the paired study design. The relative condition index of the test fish in comparison to the shoals and day of trial were included as covariates in all models to control for potential bias due to physical condition or time effects (see Table 1). First, test fish sex, shoal fish sex, predation risk (low or high), and an interaction between these three variables were used as explanatory variables. As we found a significant three-way interaction, data analysis was performed separately for each test fish sex; i.e. shoal fish sex, predation risk (low or high) and the interaction between both were used as explanatory variables. In addition, data were subdivided by predation risk (low or high); here test and shoal fish sex and the interaction between both were used as explanatory variables. Based on these findings, models were run separately for low and high predation risk trials both for males and females, with only shoal fish sex as the explanatory variable (see also Table 1 for an overview of fitted models). In all models, explanatory variables were stepwise removed in the order of statistical relevance (e.g. Engqvist, 2005). Tests of statistical significance were based on likelihood ratio tests. The residuals of the best explaining models were tested for normal distribution using the Shapiro-Wilk test. All given p-values were based on two-tailed tests, and the level of significance was set at 0.05.

3. Results

There was a significant three-way interaction effect of test fish sex, shoal fish sex, and predation risk on shoal choice behavior with regard to absolute association time (Fig. 2, Table 1 (model 1)), indicating that test fish shoal with the opposite sex within low predation risk trials and with same-sex shoals within high predation risk trials (Fig. 2).

Within male test fish, there was a significant two-way interaction effect of shoal fish sex and predation risk on shoal choice (Fig. 2, Table 1 (model 2a)). In detail, under high predation risk, males spent significantly more time shoaling with other males whereas under low predation risk males showed a non-significant tendency to spend more time shoaling with females (Fig. 2, Table 1 (model 3a&b)). Although there was no significant two-way interaction effect of shoal fish sex and predation risk on shoal choice behavior in females (Fig. 2, Table 1

Table 1
Results of lme testing the influence of test fish sex, shoal fish sex, and predation risk on absolute time spent shoaling with each shoal (dependent variable). Explanatory variables were stepwise removed from all models in the order of statistical relevance. Significant values ($P < 0.05$) are indicated in bold, trends (P between 0.05 and 0.1) in italics, and x indicates interaction term.

Model (sample size)			Explanatory variables	Δdf	χ^2	*P*
1.		Full data set (N = 40)	Test fish sex x Shoal fish sex x Predation risk	1	13.176	**0.010**
2.	a)	Male test fish (N = 20)	Shoal fish sex x Predation risk	1	5.418	**0.020**
	b)	Female test fish (N = 20)	Shoal fish sex x Predation risk	1	1.377	0.241
			Shoal fish sex	1	0.286	0.593
			Predation risk	1	5.179	**0.023**
	c)	Low predation risk (N = 20)	Shoal fish sex x Test fish sex	1	2.588	0.108
			Shoal fish sex	1	0.002	0.968
			Test fish sex	1	8.843	**0.003**
	d)	High predation risk (N = 20)	Shoal fish sex x Test fish sex	1	3.353	*0.068*
3.	a)	Male test fish under low predation risk (N = 10)	Shoal fish sex	1	1.265	0.261
	b)	Male test fish under high predation risk (N = 10)	Shoal fish sex	1	4.901	**0.027**
	c)	Female test fish under low predation risk (N = 10)	Shoal fish sex	1	2.965	*0.085*
	d)	Female test fish under high predation risk (N = 10)	Shoal fish sex	1	0.198	0.656

In each model trial number was included as a random factor and never removed to control for the paired study design. Relative condition index of the test fish in comparison to the shoals and day of trial were included as covariates in all models but had no significant influence on shoal choice behavior (all $P > 0.462$ and $P > 0.163$, respectively).

(model 2b)), a similar pattern to that shown by males was found; females tended to shoal with the opposite sex within low predation risk and with same-sex shoals within high predation risk trials (Fig. 2, Table 1 (model 3c&d)). The results further show that irrespective of shoal fish sex, female test fish spent significantly more absolute time shoaling under high predation risk (Fig. 2, Table 1 (model 2b)).

Moreover, under low predation risk there was no significant two-way interaction effect between shoal and test fish sex on shoaling behavior (Fig. 2, Table 1 (model 2c)), and independent of shoal fish sex, test fish sex had a significant influence on shoaling preference, i.e. males spent significantly more time in front of both stimulus shoals (Fig. 2, Table 1 (model 2c)). In the high predation risk trials we found a tendency for a two-way interaction effect between shoal fish sex and test fish sex on shoal choice (Fig. 2, Table 1 (model 2d)). Males significantly preferred same-sex shoals whereas females spent less time shoaling with males, but not significantly so (Fig. 2, Table 1 (model 3b& d)).

Generally, males were more active when assessing shoals than females, as measured by entrances and exits from preference zones (Fig. 3; independent samples t-test: $t = -3.877$, $N_{\text{males}} = 20$, $N_{\text{females}} = 20$, $P < 0.001$). In addition, males tended to spend more absolute time shoaling than females (two-sample Wilcoxon test: $W = 128$, $N_{\text{males}} = 20$, $N_{\text{females}} = 20$, $P = 0.053$). Irrespective of test fish sex, activity level and absolute time shoaling did not differ between low and high

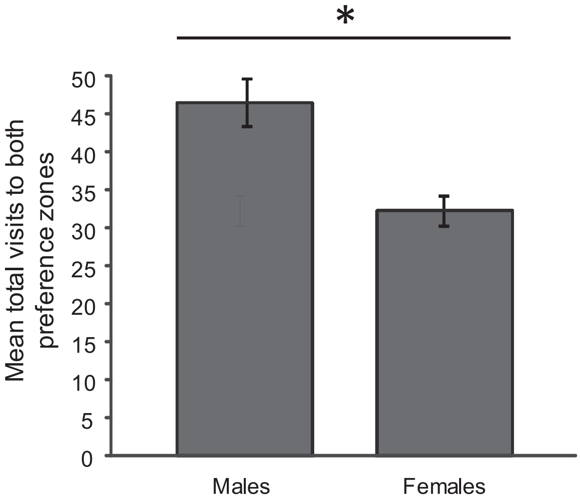

Fig. 3. Mean total visits of female and male test fish to both preference zones. Error bars represent standard error. * $P < 0.001$.

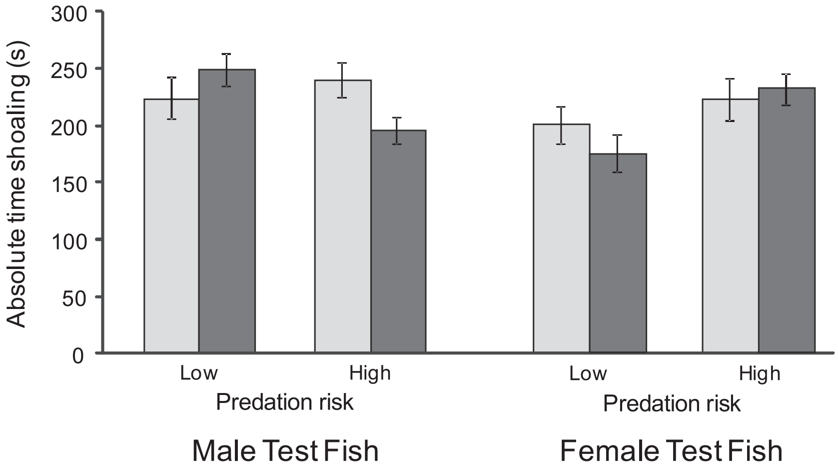

Fig. 2. Mean absolute time (s) of male and female test fish shoaling with males (light grey) and females (dark grey) in low and high predation risk trials. See also Table 1 for statistics. Error bars represent standard error.

predation risk (two-sample Wilcoxon test: both $W = 188$, $N_{\text{low predation risk}} = 20$, $N_{\text{high predation risk}} = 20$, both $P \geq 0.755$).

As individuals were size matched, there was no significant difference in standard length among shoal males and females (paired t-test: $t = 0.673$, $N = 40$, $P = 0.505$) and test males and females (two-sample Wilcoxon test: $W = 256$, $N_{\text{males}} = 20$, $N_{\text{females}} = 20$, $P = 0.124$). However, the condition index was significantly higher for males than females in both test fish (independent samples t-test: $t = -4.899$, $N_{\text{males}} = 20$, $N_{\text{females}} = 20$, $P < 0.001$) and shoal fish (paired t-test: $t = -11.256$, $N = 40$, $P < 0.001$). When relative condition index of the test fish in comparison to the shoals was included as covariates in all models, no significant influence could be determined overall (all $P > 0.462$, see also Table 1 and Data analyses for details).

4. Discussion

The results of this study indicate that sub-adult, threespine stickleback males and females make different shoaling decisions depending upon the perceived predation threat level and shoal fish sex. Sub-adult males preferred to shoal with other males when predation risk was high. If the sexes at the monochromatic sub-adult stage differ behaviorally, it would be advantageous to sexually segregate while shoaling under high predation risk to increase the confusion effect and minimize the costs of behavioral asynchrony (Ruckstuhl, 2007). The cost of synchronizing activity is lowest in groups composed of individuals of the same size, sex, and age (Conradt and Roper, 2000). Our experiment controlled for size and age, with sex remaining as a possible factor to influence behavioral synchrony. We found differences between the sexes in activity, supporting the activity synchronization and activity budget hypotheses which state that mixed-sex groups are less stable due to differences in activity (Conradt and Roper, 2000; Ruckstuhl, 2007). Group stability and cohesion would be exceptionally important when predation risk is high, resulting in the significant preference of males for male shoals observed under high predation risk. Under low predation threat levels, on the other hand, males did not demonstrate a preference for either shoal type, although there was a trend towards shoaling with females. At least two different factors might have influenced this result. First, fish may be less aggressive in the presence of predators (e.g., sexually mature male guppies (Kelly and Godin, 2001); non-reproductive convict cichlids (Wisenden and Sargent, 1997; Kim et al., 2004); bluntnose minnows (Morgan, 1988)). If aggression among same-sex sub-adults is higher under a low predation threat, this might result in some sub-adults shoaling with the opposite sex to reduce the cost of aggression. Second, sexually mature zebrafish and mosquitofish males have been shown to prefer to shoal with females, although shoaling was not tested in the presence of predators (Ruhl and McRobert, 2005; Agrillo et al., 2008). As the sub-adults in the present study were nearing the reproductive stage, they may have been interested in the opposite sex, which reduced the level of sexual segregation.

As with males, females tended to shoal with the opposite sex under low predation risk and with same-sex shoals under high predation risk, although this was not significant. Griffiths et al. (2014) reported that non-reproductive European minnow males showed same-sex association preferences whereas females did not when allowed to associate freely under more natural, low-risk conditions. Overall, our results suggest that monochromatic sub-adult sticklebacks are able to discriminate the sex of non-reproductive conspecifics and that shoaling decisions depend on predation risk. In addition, sub-adult females, but not males, did significantly increase their time spent shoaling when predator cues were present, likely taking advantage of shoaling as a means to reduce risk when odor cues suggested that predation was an imminent threat. This result could be one more manifestation of intersexual differences in boldness in sub-adult sticklebacks, as discussed below.

Regardless of the presence or absence of predator cues, sub-adult males and females behaved differently when shoaling. Males were more active when making shoaling decisions than females. These results support observations that males and females have asynchronous activity, leading to the sexual segregation observed. The higher activity of males than females is in agreement with previous studies (e.g., guppies (Griffiths and Magurran, 1998; Croft et al., 2003); minnows (Griffiths et al., 2014)) and may be due to the higher body condition found in males than females in this study. Males and females were kept together in a large tank before the experiment, and no food remained after feeding. Males may have been more aggressive during feeding than females and acquired more food, although aggression levels of juvenile male and female sticklebacks until shortly before the reproductive stage are similar (Bakker, 1986). Larger sticklebacks are preferred by predators thereby marginally increasing the predation risk of the males in our study, which could result in the behavioral and activity differences seen while shoaling (Külling and Milinski, 1992). Furthermore, if competitive abilities vary with size or sex, under low-predation trials males may have been selecting poorer competitors by shoaling with females, and females may have been avoiding competition by shoaling less (Metcalfe and Thomson, 1995; Ward et al., 2006). Moreover, intersexual differences in activity levels could also be a result of differences in personality types, which have been shown to significantly affect activity in great tits (*Parus major*) (Aplin et al., 2013), Trinidadian guppies (Croft et al., 2009), and threespine sticklebacks (Pike et al., 2008). In particular, male sub-adult sticklebacks are bolder and make faster decisions than females (King et al., 2013; Mamuneas et al., 2015). Bolder sticklebacks have higher exploratory tendencies in a novel environment (such as the experimental tank), are more active in the presence of a predator, and are more prone to shoal (Dzieweczynski and Crovo, 2011; see similar results for guppies, Barbosa et al., 2016). Any or all of these variables could have affected our discovery that sub-adult males are more active than females.

The behavior of the shoals cannot be excluded as a possible influence on the test fish behavior as the shoals could also respond to the predator cues and test fish behavior. The female and male shoals may have behaved differently, and the female and male test fish may have responded to this behavior in different ways. If the activity level of male and female shoals changed under high predation risk, there would be a potential for assortment by activity level due to the response of the shoal to the predator cue (Rehnberg and Smith, 1988). Additionally, the absolute size of the shoal may have affected the test fish's behavior. For example, Morgan (1988) demonstrated that bluntnose minnows (*Pimephales notatus*) in small shoals (3 or 5 individuals) left the shoal more often (straggled) in the presence of a predator versus no predator. The small shoal size used in this study (4 individuals), therefore, may have influenced the activity levels of males. We did not examine the behavior of the shoal fish, and the possible dynamic and synergistic interactions between test fish and potential shoal mates may influence shoaling decisions and could be an interesting avenue for future research.

Studies involving sexual segregation have long been confined to species with a strong sexual dimorphism (Ruckstuhl, 2007). However, our results suggest that sex differences when grouping may be common among species with a monomorphic, or more specifically monochromatic, non-reproductive stage due to sex differences in behavior, activity budgets, predation risk, and reproductive strategies. Further investigations should be carried out under natural conditions to determine if these preferences lead to sexual segregation in the wild, such as by determining the sex composition of complete wild shoals. It is unclear whether shoaling preferences other than the sex composition of shoals such as familiarity and/or relatedness (e.g. Frommen et al., 2007), shoal size (e.g. Mehlis et al., 2015), and shoal density (e.g. Frommen et al., 2009) may overrule sexual segregation. Uncovering the evolution of sexual segregation requires investigating sexual segregation across the spectrum from monomorphic to sexually dimorphic species (Ruckstuhl, 2007) and of dimorphic and monomorphic stages of dimorphic species. Further studies should emphasize the importance of including sex identification in behavioral studies with species that are monomorphic during the non-reproductive stage to determine whether behavioral differences exist between the sexes in the absence of physical differences.

5. Conclusions

Although non-reproductively active threespine sticklebacks are monochromatic, males and females behaved differently when given the option to shoal with males or females under low or high predation risk. This indicates that behavioral differences between males and females can be responsible for sexual segregation even in the absence of a sexual dichromatism and that sexual segregation is also dependent on environmental factors such as threat of predation.

Ethical approval

This study conforms to the Animal Behavior Society guidelines for the use of animals in research. The parental generation was caught with permission and kept under optimal conditions. Holding and rearing conditions were approved by the City of Bonn Amt für Umwelt, Verbraucherschutz und Lokale Agenda, § 11 Abs. 1 TierSchG. Spine clipping was necessary to non-invasively identify the sex prior to the experiment, and the quick and simple procedure ensured minimal negative handling impacts on the fish. Spine cutting is commonly done in stickleback studies and is not harmful to the fish (it only temporarily increases immune responses: Wedekind and Little, 2004; Henrich et al., 2014). No behavioral changes due to this procedure were observed. The number of study individuals was reduced by reusing shoal fish throughout the experiment. After the experiment, fish were returned to the original holding tank and used as breeding stock for further experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability

Original datasets associated with this paper are available from the corresponding author on reasonable request.

Acknowledgments

We thank the Bakker research group for discussion and Timo Thünken for statistical advice. We thank Dagmar Wenzel for assistance with molecular sexing, Simon Vitt for breeding the F1 generation, and Jan Hottentot for catching the parental generation in the field. We would also like to thank Deborah McLennan, and one anonymous referee for thoughtful comments on an earlier version of the manuscript.

References

Agrillo, C., Dadda, M., Serena, G., 2008. Choice of female groups by male mosquitofish (*Gambusia holbrooki*). Ethology 114, 479–488.

Aplin, L.M., Farine, D.R., Morand-Ferron, J., Cole, E.F., Cockburn, A., Sheldon, B.C., 2013. Individual personalities predict social behaviour in wild networks of great tits (*Parus major*). Ecol. Lett. 16, 1365–1372.

Arnold, K.E., Adam, A., Orr, K.J., Griffiths, R., Barber, I., 2003. Sex-specific survival and parasitism in three-spined sticklebacks: seasonal patterns revealed by molecular analysis. J. Fish Biol. 63, 1046–1050.

Bakker, T.C.M., 1986. Aggressiveness in sticklebacks (*Gasterosteus aculeatus* L.): a behaviour-genetic study. Behaviour 98, 1–144.

Bakker, T.C.M., Giger, T., Frommen, J.G., Largiadèr, C.R., 2017. Rapid molecular sexing of three-spined sticklebacks, *Gasterosteus aculeatus* L., based on large Y-chromosomal insertions. J. Appl. Genet. 58, 401–407.

Barber, I., 2003. Parasites and size-assortative schooling in three-spined sticklebacks. Oikos 101, 331–337.

Barbosa, M., Camacho-Cervantes, M., Ojanguren, A.F., 2016. Phenotype matching and early social conditions affect shoaling and exploration decisions. Ethology 122, 171–179.

Bell, M.A., Foster, S.A., 1994. The Evolutionary Biology of the Threespine Stickleback. Oxford University Press, New York.

Bolger, T., Connolly, P.L., 1989. The selection of suitable indices for the measurement and analysis of fish condition. J. Fish Biol. 34, 171–182.

Cano, J.M., Makinen, H.S., Merila, J., 2008. Genetic evidence for male-biased dispersal in the three-spined stickleback (*Gasterosteus aculeatus*). Mol. Ecol. 17, 3234–3242.

Conradt, L., Roper, T.J., 2000. Activity synchrony and social cohesion: a fission-fusion model. Proc. R. Soc. B 267, 2213–2218.

Croft, D.P., Arrowsmith, B.J., Bielby, J., Skinner, K., White, E., Couzin, I.D., Magurran, A.E., Ramnarine, I., Krause, J., 2003. Mechanisms underlying shoal composition in the Trinidadian guppy, *Poecilia reticulata*. Oikos 100, 429–438.

Croft, D.P., Botham, M.S., Krause, J., 2004. Is sexual segregation in the guppy, *Poecilia reticulata*, consistent with the predation risk hypothesis? Environ. Biol. Fish. 71, 127–133.

Croft, D.P., Krause, J., Darden, S.K., Ramnarine, I.W., Faria, J.J., James, R., 2009. Behavioural trait assortment in a social network: patterns and implications. Behav. Ecol. Sociobiol. 63, 1495–1503.

Dzieweczynski, T.L., Crovo, J.A., 2011. Shyness and boldness differences across contexts in juvenile three-spined stickleback *Gasterosteus aculeatus* from an anadromous population. J. Fish. Biol. 79, 776–788.

Engqvist, L., 2005. The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. Anim. Behav. 70, 967–971.

Etinger, A., Lebron, J., Palestis, B.G., 2009. Sex-assortative shoaling in zebrafish (*Danio rerio*). Bios 80, 153–158.

Frommen, J.G., Bakker, T.C.M., 2004. Adult three-spined sticklebacks prefer to shoal with familiar kin. Behaviour 141, 1401–1409.

Frommen, J.G., Mehlis, M., Brendler, C., Bakker, T.C.M., 2007. Shoaling decisions in three-spined sticklebacks (*Gasterosteus aculeatus*) – familiarity, kinship and inbreeding. Behav. Ecol. Sociobiol. 61, 533–539.

Frommen, J.G., Hiermes, M., Bakker, T.C.M., 2009. Disentangling the effects of group size and density on shoaling decisions of three-spined sticklebacks (*Gasterosteus aculeatus*). Behav. Ecol. Sociobiol. 63, 1141–1148.

Frommen, J.G., Herder, F., Engqvist, L., Mehlis, M., Bakker, T.C.M., Schwarzer, J., Thünken, T., 2011. Costly plastic morphological responses to predator specific odour cues in three-spined sticklebacks (*Gasterosteus aculeatus*). Evol. Ecol. 25, 641–656.

Griffiths, S.W., Magurran, A.E., 1997. Familiarity in schooling fish: how long does it take to acquire? Anim. Behav. 53, 945–949.

Griffiths, S.W., Magurran, A.E., 1998. Sex and schooling behaviour in the Trinidadian guppy. Anim. Behav. 56, 689–693.

Griffiths, S.W., Orpwood, J.E., Ojanguren, A.F., Armstrong, J.D., Magurran, A.E., 2014. Sexual segregation in monomorphic minnows. Anim. Behav. 88, 7–12.

Harcourt, J.L., Sweetman, G., Johnstone, R.A., Manica, A., 2009. Personality counts: the effect of boldness on shoal choice in three-spined sticklebacks. Anim. Behav. 77, 1501–1505.

Henrich, T., Hafer, N., Mobley, K.B., 2014. Effects of VIE tagging and partial tissue sampling on the immune response of three-spined stickleback *Gasterosteus aculeatus*. J. Fish Biol. 85, 965–971.

Hoare, D.J., Krause, J., Peuhkuri, N., Godin, J.-G.J., 2000. Body size and shoaling in fish. J. Fish Biol. 57, 1351–1366.

Huntingford, F.A., Ruiz-Gomez, M.L., 2009. Three-spined sticklebacks (*Gasterosteus aculeatus*) as a model for exploring behavioural biology. J. Fish Biol. 75, 1943–1976.

Johnson, M.B., Lafferty, K.D., van Oosterhout, C., Cable, J., 2011. Parasite transmission in social interacting hosts: monogenean epidemics in guppies. PLoS One 6, e22634.

Kelley, J.L., Graves, J.A., Magurran, A.E., 1999. Familiarity breeds contempt in guppies. Nature 401, 661–662.

Kelly, C.D., Godin, J.-G.J., 2001. Predation risk reduces male-male sexual competition in the Trinidadian guppy (*Poecilia reticulata*). Behav. Ecol. Sociobiol. 51, 95–100.

Kim, J., Brown, G.E., Grant, J.W.A., 2004. Interactions between patch size and predation risk affect competitive aggression and size variation in juvenile convict cichlids. Anim. Behav. 68, 1181–1187.

King, A.J., Fuertbauer, I., Mamuneas, D., James, C., Manica, A., 2013. Sex-differences and temporal consistency in stickleback fish boldness. PLoS One 8, e81116.

Kohn, G.M., King, A.P., Scherschel, L.L., West, M.J., 2011. Social niches and sex assortment: uncovering the developmental ecology of brown-headed cowbirds, *Molothrus ater*. Anim. Behav. 82, 1015–1022.

Krause, J., Ruxton, G.D., 2002. Living in Groups. Oxford University Press, Oxford.

Krause, J., Godin, J.-G.J., Brown, D., 1996. Phenotypic variability within and between fish shoals. Ecology 77, 1586–1591.

Külling, D., Milinski, M., 1992. Size-dependent predation risk and partner quality in predator inspection of sticklebacks. Anim. Behav. 44, 949–955.

Landeau, L., Terborgh, J., 1986. Oddity and the ‘confusion effect’ in predation. Anim. Behav. 34, 1372–1380.

Magurran, A.E., 1998. Population differentiation without speciation. Philos. Trans. R. Soc. B 353, 275–286.

Mamuneas, D., Spence, A.J., Manica, A., King, A.J., 2015. Bolder stickleback fish make faster decisions, but they are not less accurate. Behav. Ecol. 26, 91–96.

McGee, M.D., Wainwright, P.C., 2013. Sexual dimorphism in the feeding mechanism of threespine stickleback. J. Exp. Biol. 216, 835–840.

McRobert, S.P., Bradner, J., 1998. The influence of body coloration on shoaling preferences in fish. Anim. Behav. 56, 611–615.

Mehlis, M., Thünken, T., Bakker, T.C.M., Frommen, J.G., 2015. Quantification acuity in spontaneous shoaling decisions of three-spined sticklebacks. Anim. Cogn. 18, 1125–1131.

Metcalfe, N.B., Thomson, B.C., 1995. Fish recognize and prefer to shoal with poor competitors. Proc. R. Soc. B 259, 207–210.

Modarressie, R., Rick, I.P., Bakker, T.C.M., 2006. UV matters in shoaling decisions. Proc. R. Soc. B 273, 849–854.

Morgan, M.J., 1988. The effect of hunger, shoal size, and the presence of a predator on shoal cohesiveness in bluntnose minnows, *Pimephales notatus* Rafinesque. J. Fish Biol. 32, 963–971.

Peuhkuri, N., 1998. Shoal composition, body size and foraging in sticklebacks. Behav. Ecol. Sociobiol. 43, 333–337.

Pike, T.W., Samanta, M., Lindström, J., Royle, N.J., 2008. Behavioural phenotype affects social interactions in an animal network. Proc. R. Soc. B 275, 2515–2525.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Team, R.C., 2016. nlme: linear and nonlinear mixed effects models. R Package Version 3.1-128. . http://CRAN.R-project.org/package=nlme.

Ranta, E., Lindström, K., 1990. Assortative schooling in three-spined sticklebacks? Ann. Zool. Fenn. 27, 67–75.

R Development Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Version 3.0.2. R Foundation for Statistical Computing, Austria, Vienna. https://cran.r-project.org/.

Rehnberg, B.G., Smith, R.J.F., 1988. The influence of alarm substance and shoal size on the behavior of zebra danios, *Brachydanio rerio* (Cyprinidae). J. Fish Biol. 33, 155–163.

Richards, E.L., van Oosterhout, C., Cable, J., 2010. Sex-specific differences in shoaling affect parasite transmission in guppies. PLoS One 5, e13285.

Romey, W.L., Galbraith, E., 2008. Optimal group positioning after a predator attack: the influence of speed, sex, and satiation within mobile whirligig swarms. Behav. Ecol. 19, 338–343.

Ruckstuhl, K.E., 2007. Sexual segregation in ungulates: proximate and ultimate causes. Integr. Comp. Biol. 47, 245–257.

Ruckstuhl, K.E., Clutton-Brock, T.H., 2005. Sexual segregation and the ecology of the two sexes. In: Ruckstuhl, K.E., Neuhaus, P. (Eds.), Sexual Segregation in Vertebrates: Ecology of the Two Sexes. Cambridge University Press, Cambridge, pp. 3–10.

Ruckstuhl, K.E., Neuhaus, P., 2002. Sexual segregation in ungulates: a comparative test of three hypotheses. Biol. Rev. 77, 77–96.

Ruhl, N., McRobert, S.P., 2005. The effect of sex and shoal size on shoaling behaviour in *Danio rerio*. J. Fish Biol. 67, 1318–1326.

Snekser, J.L., Ruhl, N., Bauer, K., McRobert, S.P., 2010. The influence of sex and phenotype on shoaling decisions in zebrafish. Int. J. Comp. Psychol. 23, 70–81.

Theodorakis, C.W., 1989. Size segregation and the effects of oddity on predation risk in minnow schools. Anim. Behav. 38, 496–502.

Velando, A., Costa, M.M., Kim, S.Y., 2017. Sex-specific phenotypes and metabolism-related gene expression in juvenile sticklebacks. Behav. Ecol. 28, 1553–1563.

Ward, A.J.W., Thomas, P., Hart, P.J.B., Krause, J., 2004. Correlates of boldness in three-spined sticklebacks (*Gasterosteus aculeatus*). Behav. Ecol. Sociobiol. 55, 561–568.

Ward, A.J.W., Webster, M.M., Hart, P.J.B., 2006. Intraspecific food competition in fishes. Fish. Fish. 7, 231–261.

Wearmouth, V.J., Sims, D.W., 2008. Sexual segregation in marine fish, reptiles, birds and mammals: behaviour patterns, mechanisms and conservation implications. Adv. Mar. Biol. 54, 107–170.

Wedekind, C., Little, T.J., 2004. The clearance of hidden cestode infection triggered by an independent activation of host defense in a teleost fish. J. Parasitol. 90, 1329–1331.

Wisenden, B.D., Sargent, R.C., 1997. Antipredator behaviour and suppressed aggression by convict cichlids in response to injury-released chemical cues of conspecifics but not to those of an allopatric heterospecific. Ethology 103, 283–291.