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Territoriality and nest competition in the two-spotted goby (*Pomatoschistus flavescens*)

Master's thesis in Biology – Ecology, Behaviour, Evolution and
Biosystematics

Supervisor: Trond Amundsen, Irja Ida Ratikainen

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Trondheim, 2019

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Abstract

Intra-sexual competition plays an important role in the evolutionary process of sexual selection. Male-male competition over resources that are essential for mating success may prevent unsuccessful competitors from breeding. The intensity of competition is influenced by the availability, quality, and distribution of such resources. The spatial distribution of resources may facilitate monopolisation, limit the density of breeders and skew the reproductive success among individuals in a population. In some species, one such resource is favourable nests sites, which males compete for, occupy and defend against intruders. The aim of this study was to test the extent to which nest spacing, and specifically inter-nest distance, induces intra-sexual competition (territorial aggression). In order to address this question I focused on how distance between pairs of nests affects nest occupancy, mating success and asynchrony in breeding of male two-spotted gobies (*Pomatoschistus flavescens*), a small fish with a resource defence based breeding system and uniparental care provided by males. Results show that shorter inter-nest distance did not affect nest occupancy, mating success or asynchrony in breeding of males and does not appear to intensify male-male competition. These results suggest that nest spacing does not limit the density of breeding males. However, further studies are required to understand the effect of nest spacing on the intensity of sexual selection. The high occupancy rates, irrespective of distance, suggest that the artificial nests are favourable nesting sites. Although potential natural nesting sites for the two-spotted gobies seem to be abundant in the studied area, high-quality nests might be in limited supply.

Introduction

Sexual selection occurs when the variation in mating success among individuals in a population is nonrandom, as the result of intra-sexual competition and/or intersexual mate choice (Darwin 1871). The intra-sexual competition determines an individual's access to resources essential for reproduction as well as to mating partners (Andersson 1994; Clutton-Brock 2007; Clutton-Brock 2009). Intra-sexual competition occurs both in females and males (Andersson 1994; Clutton-Brock 2007, 2009), but commonly is more intense among the males (Andersson 1994; Miller 2013). Competition among males, in the form of visual displays and/or physical aggression, determines which individuals within a population have access to and can monopolise these resources by excluding competitors (e.g. Ahnesjö et al. 2001). The monopolisation of resources by a fraction of the male population can prevent or limit subordinate individuals' mating attempts and therefore result in a skewed distribution of successful mating among males (Emlen and Oring 1977; Warner 1980).

The strength of intra-sexual competition and hence sexual selection may vary among species, populations and even within populations over time (McLain 1982; Forsgren et al. 1996; Mobley and Jones 2007, 2009; Grant and Grant 2002; Ahnesjö et al. 2008; Siepielski et al. 2009; Miller and Svensson 2014). There are several factors that may affect the intensity of competition. The operational sex ratio (OSR), the ratio between sexually active males and females (Emlen and Oring 1977; Kvarnemo and Ahnesjö 1996), is considered to be one of these factors (but see Kokko and Monaghan, 2001), and it may have an impact on both male-male competition and mate choice (Emlen and Oring 1977; Jirotkul 1999; Clutton-Brock 2007; Weir et al. 2011). The relation between OSR and the strength and direction of mating competition has been shown in a wide variety of taxa of mammals (e.g. Schwagmeyer and Brown 1983), birds (e.g. Colwell and Oring 1988), insects (e.g. Cratsley and Lewis 2005) and fish (e.g. Vincent et al. 1994; Borg et al. 2002; Forsgren et al. 2004). In species or populations with male-biased OSRs, competition among males is expected to be more intense than in species or populations with a more balanced OSR. Since in male-biased OSRs there are disproportionately more male competitors and relatively few receptive females, females can afford to be selective thanks to the excess of potential mates (e.g. Lawrence 1986; Enders 1993; Krupa and Sih 1993; Jormalainen et al. 1994; Dick and Elwood 1996; Clutton-Brock et al. 1997; Balshine-Earn 1996). By contrast, when OSR is strongly female-biased, females are the ones facing shortage of potential mates and female-female competition may rise.

One of the main factors that may create a bias in the OSR is the distribution and availability of breeding resources. The availability and spatial distribution of breeding resources, such as suitable nest sites, affects the number of successful breeders in a given reproductive season and potentially has a significant impact on the intensity of competition (Village 1983; Grant 1993; Forsgren et al. 1996; Reynolds 1996; Kwiatkowski and Sullivan 2002; Lehtonen and Lindström 2004; Larison 2007). In some species, competition for access to finite breeding resources may generate extensive variation in mating success and consequently sexual selection (Emlen and Oring 1977; Ahnesjö et al. 2001; Shuster and Wade 2003). In species where male nest ownership is a prerequisite for successful reproduction, a shortage of suitable nest sites is associated with stronger male-male competition, whereas an abundance leads to a more relaxed resource competition but more intense intersexual selection (Nellbring 1986; Forsgren et al. 1996). The distribution of nest sites may also have a profound effect on the competition among males, since aggregated suitable nest sites can be monopolised by a subset of males in the population and exclude the less successful competitors from breeding (Forsgren et al. 1996; Oliveira et al. 1999; Ahnesjö et al. 2001; Clutton-Brock 2007; Mück et al. 2013). Consequently, nest monopolisation, as a result of scarcity and patchiness, may trigger changes in the degree of male-male competition. However, fewer studies have focused on the degree to which inter-nest distance results in monopolisation and thus intense intra-sexual competition.

Resource defence breeding systems are widespread among fish, where typically males defend a spawning site or build a nest in which they attract multiple females to lay their eggs (Forsgren et al. 2002). This type of mating system is common among gobies (Gobiidae), sticklebacks (Gasterosteidae), labrids (Labridae) and damselfish (Pomacentridae) (Forsgren et al. 2002). In these species, the territorial behaviour of males, which defend the area surrounding the nests against intruders, contributes to intra-sexual competition. For example, in the common goby (*Pomatoschistus microps*), nest-holders are known to display agonistic behaviours, such as chasing and physical attacks, towards other males approaching their nest (Magnhagen 1995). Therefore, territoriality might prevent or delay the establishment and thus breeding of other males close to a nest owner's territory and reduce the occupancy of adjacent suitable nest sites. Since males of these species invest considerable time and energy in aggressive interactions and nest guarding (e.g. Gross 1980; Sargent 1981, 1982; Unger 1983), there might also be a trade-off between territoriality and courtship (e.g. see Kangas and Lindström 2001). The allocation of time and energy between nest defence, aggression and mating may affect the brood size. Males that engage more in territorial aggression spend

less time in female courtship and most likely attract fewer females to spawn in their nests. In a laboratory study in the two-spotted goby, increased male-male aggression due to dense spacing of nests resulted in reduced nest occupancy and brood size (Mück et al. 2013). Competing males may also engage in courtship interference, which results in unsuccessful mating attempts and may lead to a smaller brood size. In areas where nests are aggregated and territories are dense or overlapping, increased male-male interaction could lead to more frequent courtship interference (Kangas and Lindström 2001). Dominant males might often interrupt the courtship of females by subordinates (Kangas and Lindström 2001; Wong 2004), leading to a skewed mating success (Östlund-Nilsson and Nilsson 2000). Competitively inferior males with fewer successful mating attempts might attract fewer females and receive fewer eggs per breeding cycle, resulting in a reduced brood size. In a small fresh-water goby (*Padogobius martensi*), nest intrusion and courtship interference by competitor males occurred frequently during spawning (Bisazza et al. 1989), especially when nests were located in close proximity to the natural breeding areas (Marconanto et al. 1989). Consequently, increased competition as the result of nest distribution may not only affect the success and timing of breeding of the males but their brood size as well.

However, the degree to which nest distribution affects male-male competition remains largely unknown. A shorter inter-nest distance may facilitate nest monopolisation by a subset of the male population and intensify intra-sexual competition. The aim of this study was to test the extent to which spacing of favourable nest substrates induces male-male competition (territorial behaviour) preventing males from breeding in close proximity to each other, and hence limiting the density of breeding males. It was also tested to what extent male-male competition affects the mating success and the breeding synchrony of the males. In order to address these questions, I focused on how the distance between pairs of nests affects: (1) nest occupancy (2) brood size and (3) asynchrony in breeding of males in neighbouring nests. The species in this case was the two-spotted goby, a small fish with resource defence based breeding system and uniparental care provided by males (Forsgren et al. 2004; Mobley et al. 2009; Wacker et al. 2014; Monroe et al. 2016; Amundsen 2018). These characteristics along with the very dynamic sexual selection regimes in this species (Amundsen and Forsgren 2001; Forsgren et al. 2004; Myhre et al. 2012; Amundsen 2018), make the two-spotted goby a suitable model species for studies in intra-sexual competition.

Since we would expect territorial behaviour to prevent males from establishing in nearby nest sites, occupancy is predicted to increase as the inter-nest distance increases. The territorial behaviour of an already established male might delay rather than prevent the

establishment of other males in neighbouring sites. In such cases, we would expect eggs to appear in the second nest of a pair later than in the first, especially when the nests are close. Thus, the asynchrony in breeding of neighbouring males is predicted to decrease with inter-nest distance. If competition and courtship interference lead to reduced number of females that competing males successfully attract to spawn in their nests, we predict an increase in each individual male's brood size, as the inter-nest distance increases. However, asymmetry in the competitive abilities of the neighbouring nest-holders might lead to the reduction of the brood size only for the unsuccessful competitor. In that case, we would expect a smaller difference between the brood sizes of neighbouring males, as the inter-nest distance increases.

Methods

Study species

The two-spotted goby (*Pomatoschistus flavescens*) is a small, 35-55 mm in total length (Wacker et al. 2013), semi-pelagic, marine fish species. The species has recently been added to the genus *Pomatoschistus* (Thacker et al. 2018), within the family Gobiidae. The two-spotted goby inhabits kelp forests and seaweed beds and it is highly abundant along the shallow algal zone of the rocky coasts of Western Europe (Collins 1981, Miller 1986), at approximately 0 to 5 metres depth (Amundsen 2018). The two-spotted goby is probably of major importance for the wild cod, as it is one of the main prey species (Fosså 1991; Nordeide and Salvanes 1991; Steingrund and Fernö 2005). These characteristics possibly make the two-spotted goby a keystone species for the coastal ecosystems of the rocky shores of Scandinavia (Fosså 1991; Giske et al. 1991; Hop et al. 1992, 1993).

The species is sexually dimorphic with both males and females conspicuously ornamented (Amundsen and Forsgren 2001; Amundsen 2018) (Fig. 1a). Males have iridescent blue spots, enlarged brightly coloured dorsal fins and two distinctive dark spots, one at the base of the tail and one at the base of pectoral fin (Amundsen and Forsgren 2001; Wacker et al. 2013). Females have a less bright body colouration with the exception of orange-coloured bellies, which they display during courtship of males (Amundsen and Forsgren 2001; Sköld et al. 2008). Males exhibit a mating preference for the more colorful females, which indicates that the female ornamentation is also under sexual selection due to male mate choice (Amundsen and Forsgren 2001).

The two-spotted goby is a substrate brooding, mostly annual species with only a few individuals surviving a second year (Holt and Byrne 1903; Collins 1981), depending on latitude (Johnsen 1945). The reproductive season lasts from April-May until July (Johnsen 1945; Forsgren et al. 2004; Myhre et al. 2012; Wacker et al. 2013). During the breeding season, both females and males may reproduce repeatedly (Eriksen 2007, de Jong 2011) and compete for mating through courtship displays and agonistic behaviours (Amundsen and Forsgren 2001; Forsgren et al. 2004; Myhre et al. 2012). Fish of both sexes form loose foraging shoals, with range that varies from less than 10 to up to several hundred individuals (Amundsen 2018). During the breeding season, however, territorial males are usually solitary (Forsgren et al. 2004), whereas females that are ready to mate join smaller unisexual shoals or sometimes occur solitarily (Myhre et al. 2012; Amundsen 2018). Successful males occupy and defend nests, located in natural crevices, rocks, on a range of kelp (e.g. *Laminaria* sp.)

and seaweed species and empty mussels (Gordon 1983; Forsgren et al. 2004; Mobley et al. 2009; Mück et al. 2012; Wacker et al. 2014; Amundsen 2018)(Fig. 1b). Nest ownership is a prerequisite for successful breeding with the exception of individuals displaying sneaking behaviour (Utne-Palm et al. 2015), although this alternative reproductive tactic seems to be uncommon in some populations of this species (Mobley et al. 2009; Wacker et al. 2014). However, sneaking rates may vary among populations (Monroe et al. 2016). Typically successful males mate with several females that deposit clutches of eggs in their nests (Mobley et al. 2009; Wacker et al. 2014; Monroe et al. 2016). Males alone provide parental care until hatching (Skolbekken and Utne-Palm 2001). The hatching period depends on the temperature of the water and varies between 1 and 3-4 weeks (Skolbekken and Utne-Palm 2001; Bjelvenmark and Forsgren 2003; Svensson 2006; Amundsen 2018). Males have been observed to often consume some of their own eggs, a practice known as filial cannibalism (Rohwer 1978). Filial cannibalism possibly aims to increase investment in future reproduction and it is rather common among fish with parental care (Bjelvenmark and Forsgren 2003).

The proportion of receptive females and stationary males, typically the nest holders, in some populations changes over the breeding season (Forsgren et al. 2004; Myhre et al. 2012). Some studies on a Swedish population have shown that over the course of the breeding season, there is an increase in abundance of actively courting females that compete for the fewer males that are present (Forsgren et al. 2004; Myhre et al. 2012). The decline in the proportion of males was speculated to be due to the accumulated costs of parental care, competition, susceptibility to parasites and diseases, or an increased predation risk (Forsgren et al. 2004). This situation indicates a seasonal change in OSR and thus a shift of sex roles, since intense male–male competition and courtship behaviour in males is replaced by female–female competition and courtship by females (Forsgren et al. 2004). However, the sexual selection regimes seem to vary among populations across the geographical distribution of the species (Amundsen et al., unpubl. data.). These changes in intra-sexual competition and the dynamic mating system make the two-spotted goby an ideal model for studies on sexual selection.



Figure 1: (a) Female (top) and male (bottom) two-spotted goby (*Pomatoschistus flavescens*). Photo: Elisabet Forsgren, © National Academy of Sciences, USA (b) A male two-spotted goby in a natural nest on a kelp (*Laminaria* sp.). Photo: Trond Amundsen

Study area

The study was conducted in Bispøyan archipelago NW of Hitra (63°34' 17'' N, 8°28' 18'' E). It is an island complex, consisting of smaller and larger islands with more or less exposed rocky shores covered by algal vegetation and seaweed bed. The locations were selected based both on their accessibility (by boat) and the presence of suitable habitat for the studied species. They covered a wide variety of suitable habitats, representing a gradient from relatively barren rocky areas with scattered seaweed and mussels to ones with very dense vegetation, dominated by kelp (e.g. *Laminaria* sp., *Saccharina* sp.). Nests were placed along the coast of a total of 20 different islets, five in each of the four areas chosen within the Bispøyan archipelago (Fig. 2).

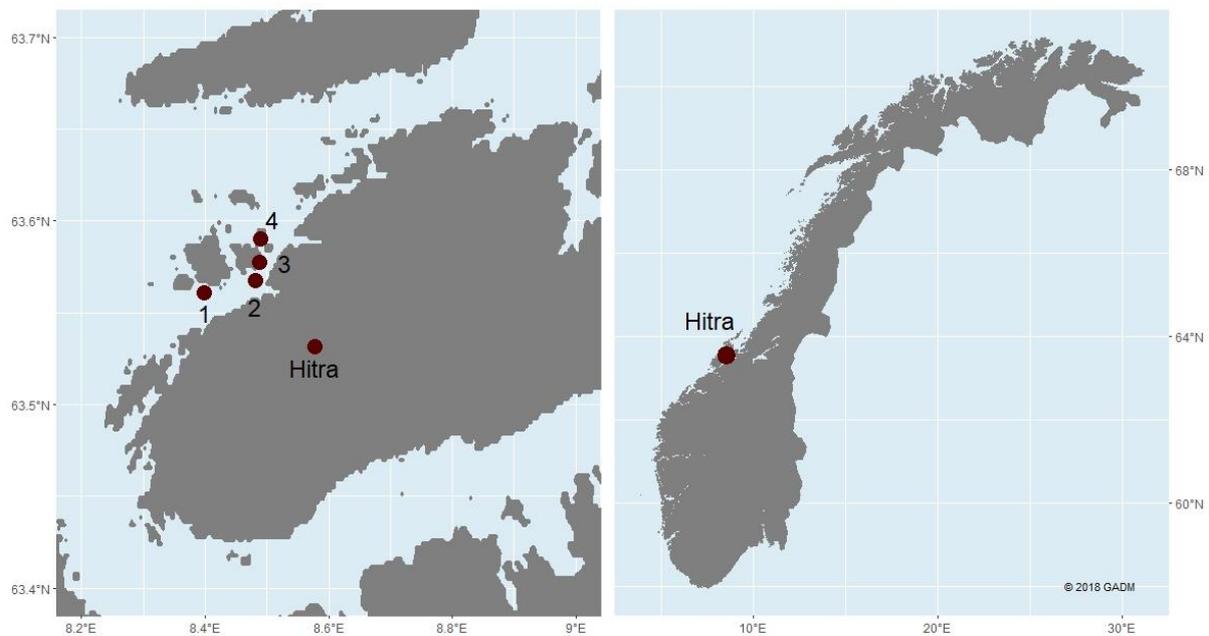


Figure 2: Left: Map of the four study areas (red dots) within the Bispøyan archipelago northwest of Hitra. Right: Map of Norway, highlighting the location of Hitra (red dot).

Experimental design

The aim of the study was to test the effect of nest distance on competition and specifically on the spacing and breeding success of male two-spotted gobies. For that purpose sets of nests were placed in the field in the study area. Each set consisted of two nests spaced at different fixed distances of 5, 20, 60, 120 and 300 cm apart (Fig. 3). The shorter distance was chosen to match the size of the fish and thus force an interaction in case males attempt to occupy the neighbouring nests. The distance of 3 metres on the other hand was deemed sufficient to minimise any interaction effects between the nests. The in between distances were similar or related to the ones used in previous experiments (Mück et al. 2013; Wacker et al. 2013). For each of the 5 different distances, a total of 20 pairs were used (10 for small and 10 for large nests). Along the shore of the islands, a full set of pairs (one for every fixed distance) was placed in randomised order, to avoid any confounding effects of the position, central or at the edges, in the selected habitat. Two different sizes, small (length: 80 mm, outer diameter: 20 mm, inner surface area: 42cm²) and large (length: 130 mm, outer diameter: 25 mm, inner surface: 84 cm²), of nests were used. The larger nests can potentially accommodate larger amount of eggs (e.g. Lindström 1988) than smaller nests used in previous studies (Mobley et al. 2009; Wacker et al. 2014, Monroe et al. 2016), allowing for higher male reproductive potential. Since owners of larger nests can receive more clutches, larger nest size increases the quality of the nest (e.g. Lindström 1992), which might affect male-male competition. Using both sizes we could improve the quality of the analysis of the brood size and test whether nest size affects occupancy rates.

The artificial nests are made of PVC tubes open in both ends. The larger tubes had twice the surface of the small ones. The inner surface of each PVC tube was covered with a removable acetate sheet with squares of 1 cm² to facilitate egg mass estimation. Acetate sheets have been successfully used to quantify brood size by Monroe et al. 2016. The PVC tubes were attached by rope to a brick in one end and a styrofoam floater in the other. The distance from the brick was adjusted so that the tube would remain in suitable height among the vegetation on which typically two-spotted gobies have their nests. The neighbouring pairs were placed at intervals of 3 or more metres apart, to secure independence and avoid any interference from each other's presence. The inter-pair intervals varied since it was deemed as more important to place the nests in suitable positions as long as the independence was secured. The nests were placed at least 50 cm below the lower tidemark to ensure that both the tube and the floater would remain submerged at all times. The floaters were used to

prevent tubes from sinking and secure a horizontal angle to avoid any confounding effects in occupancy due to tilting.

Previous studies, both in the laboratory (Amundsen and Forsgren 2001; Skolbekken and Utne-Palm 2001; Svensson et al. 2006; de Jong et al. 2009) and in the field (Forsgren et al. 2004; de Jong 2011; Monroe et al. 2016), have revealed a high rate of occupancy of such nests that seem to be favourable spawning sites. Under these circumstances we would expect males to compete for artificial nests regardless the availability of natural nesting substrates.

The setting of the artificial nests took place at the very start of the breeding season, between 30th of April and 6th of May. For each set of pairs depth, time, date, water temperature, distance of the neighbouring nests and GPS location were recorded. The experiment was repeated two times, one early and one late in the breeding season to account for potential seasonal variation in intra-sexual competition regimes. The first data collection was carried out between 28th May – 3rd June and the second later in the period between 2nd July – 9th July. At the end of the experiment in July all the tubes and bricks were collected from the study area.

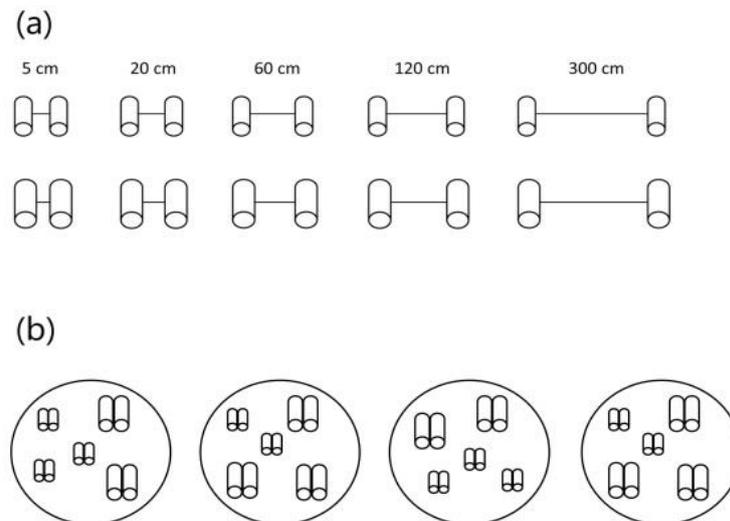


Figure 3: Illustration of the experimental design. (a) Full sets of nest pairs with 5, 20, 60, 120 and 300 cm distance. Two different nest sizes, small and large, were used. (b) Distribution of the full sets containing all the possible pair distances among the 4 areas.

Data collection

All the observations and the data collection were obtained by snorkeling. For each pair, occupancy data was recorded and the nest owners along with their clutches were collected. During the first visit in June, each nest was observed for five minutes to detect the owner's presence and any male-male (aggression or courtship interference) or male-female interaction (courtship or spawning). If there was a male present in the tube during that period it was assumed to be the nest owner. If males were absent I waited for 20 minutes for the owner to appear, after which only the clutches and the occupancy data of these nests were collected. A nest was considered occupied when a male was observed in the tube and/or it contained eggs or evidence of a hatched brood on the acetate sheet.

The males were collected either by nets or by blocking the holes of the tube on both sides and trapping the fish inside. Subsequently, both the males and the acetate sheets with the broods were transferred on land and stored in suitable boxes, labeled with the nest number, location etc. The lined sheets were replaced with new ones and the nests were returned to their previous locations, so that they could be recolonised by new males during the repetition of the experiment later in the breeding season. The males along with their broods were transported to the field station for further analysis. Their size was measured to the nearest 0.5 mm on a measuring grid.

For the calculation of brood size, two different measurements were taken. Since there were no statistically significant differences in egg density between locations, periods, or nest sizes (Table 1), the number of squares and area covered by eggs on the acetates were used for the analysis. The number of squares was visually estimated, which has been proven an accurate measurement of nest fullness in previous studies (e.g. Mück et al. 2013). The area covered with eggs was calculated (in cm^2) from photos of the broods, using ImageJ (Schneider et al. 2012). In order to assess mating success, both hatched and cannibalised parts of the brood were included for the calculations of both the number of squares and area. The two measurements were highly correlated (Pearson's correlation $r = 0.99$, $p < 0.001$) indicating high accuracy of the measurements and thus only the results for number of squares are presented. A sample ($n = 20$) of broods was measured multiple times to estimate between and within observer accuracy. Both within (Pearson's correlation $r = 0.99$, $p < 0.001$) and between (Pearson's correlation $r = 0.98$, $p < 0.001$) observers measurements showed high repeatability and high accuracy. In addition, the absolute size difference between the two broods in each pair was calculated.

The asynchrony in breeding was judged based on whether there is a difference in the developmental stage of the broods in the pairs. Since the field trips had a four-week interval period, each male had the same period to occupy the nest, attract females to lay their eggs and provide care until hatching. The range of temperatures for the different locations was from 9.4 °C in June to 12.7 °C later in July. Given that within this range of temperatures the hatching period for the eggs is two weeks (Skolbekken and Utne-Palm 2001; Bjelvenmark and Forsgren 2003), each male could care for a maximum of two consecutive broods in a 4-week period, assuming early establishment. The broods of the second breeding cycle could be identified by the presence of marks of a hatched layer underneath the eggs of the current brood. To conclude if one of the nest-owners in the pairs initiated breeding earlier than the other both criteria based on the developmental stage of the eggs and the presence/absence of previous hatched layer were used. If both broods are in the same breeding cycle (first or second), the brood with the most developed eggs was considered to be the oldest one. The male with the oldest brood was considered to initiate breeding first. If only one of the broods is from the second cycle (presence of another hatched layer underneath), it was considered to be the oldest one regardless the stage of the eggs.

Data for occupancy, asynchrony, brood size and male size were collected for both periods, early (in June) and late (in July) in the breeding season.

Table 1: Summary from Analysis of Variance (ANOVA) test for difference in the number of eggs per square (egg density) among periods, nests sizes and locations.

	Degrees of freedom	Sum of squares	Mean squares	F	P value
Nest size	1	124.0	124.0	1.060	0.314
Period	1	12.5	12.5	0.107	0.747
Location	6	1341.0	223.5	1.909	0.122
Residuals	23	2692.0	117.0		

Data analysis

All the data were analysed using R software (version 3.5.3, R Core Team 2019). The data from two locations were excluded completely from the analysis, as none or only very few nests were occupied. The observed lack of occupancy in these locations may not necessarily reflect the outcome of the intra-sexual competition and was attributed to other factors, such as the high abundance of the natural nest sites. A third location had none of the nests occupied in June and the majority occupied in July. The analyses were run twice with and without this location.

Analysis of occupancy and asynchrony

Given the binomial response of the data (double or single occupancy and asynchrony or not), generalised linear mixed effects models (GLMM) (using *glmer* in the *lme4* package in R, Bates et al. 2015), with a logit link function and binomial error distribution, were used. Distance between the nests was the main predictor and it was \log_{10} -transformed prior to the analyses. The nest size (small or large) and period (early or late) were also added as fixed factors. Two-way interactions were allowed between distance and period and location was treated as random factor in both models. In another set of models, the interaction between nest size and distance was also included.

Period was included to account for potential seasonal changes in competition regimes. Several factors that vary temporally may lead to seasonal changes in competition regimes. Such factors may be the operational sex ratio (Forsgren et al. 2004), the structural complexity of the habitat (Myhre et al. 2013), the body size of breeding males (Eigenmann 1894; Hubbs 1921; Downhower and Brown 1981; Miranda and Muncy 1987) as well as their arrival time and territory establishment in the breeding grounds (Candolin and Voigt 2003). Thus, period was added as a fixed factor with two levels in the models. These effects potentially are more profound in shorter distances and thus the interaction between distance and period was allowed.

Some studies indicate that the size of the nest is correlated with the body size of the nest owner, as larger males tend to occupy larger nests (e.g. Kvarnemo 1995; Takahashi et al. 2001). Body size is often positively correlated with the competitive ability and territory size in fish (e.g. Keeley and Grant 1995; Keeley and McPhail 1998). In the sand goby (*Pomatoschistus minutus*) there seems to be an increasing competitive ability with body size as well as behavioural differences between males of different size (Magnhagen and

Kvarnemo, 1989). In the current study, male two-spotted gobies that occupied the large nests are significantly larger than the ones that occupied the small nests (Fig. 4). For these reasons, the size of the nest was included as a fixed factor in the models. Since this effect would be more profound in shorter distances another set of models that allowed the interaction between nest size and distance was also run.

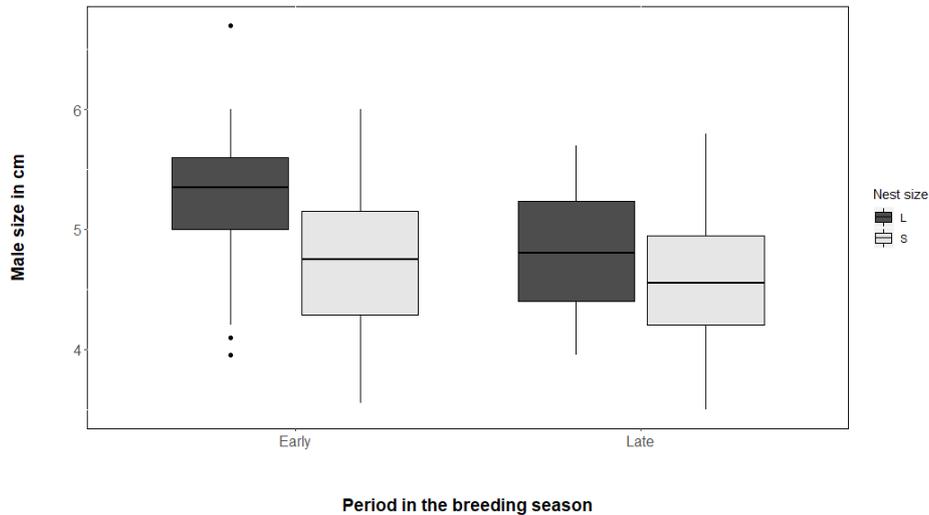


Figure 4: Differences in male nest-holders' body size in large and small nests, early and late in the breeding season. The difference in male sizes between small and large nests was statistically significant for both periods, early: mean lengths of large fish = 5.26 cm, mean length of small fish = 4.74 cm ($p < 0.0001$) and late: mean lengths of large fish = 4.82 cm, mean length of small fish = 4.58 cm, ($p = 0.009$).

Analysis of brood size

For the analysis of brood size two different linear mixed effects models (using *lmer* in the *lme4* package in R) were fitted, with individual brood size and absolute brood size difference of each pair as response variables. Distance between the nests was the main predictor and was \log_{10} -transformed prior to the analysis, for both models. Nest size and period were also included as fixed factors in both models. For the absolute brood size difference, an interaction between distance and absolute male size difference in the pairs was allowed. For the individual brood size an interaction between distance and individual male size was allowed. Nest pair number (nested within locations) was included as an additional

random factor to account for the non-independence of the two male and brood sizes of the nest holders in the pairs. Location was treated as a random factor.

One of the main assumptions of this model is that the male considered to be the nest holder is also the owner of the brood. This assumption was based on studies on the parasitic spawning in two-spotted goby populations that report generally low rates of eggs fertilised by sneaking males (Mobley et al. 2009; Monroe et al. 2016; Wacker et al. 2014).

Except for distance, male size (both individual and the difference), nest size (small and large) and period (early and late) were included to test their effects on some of the remaining variation in brood size. Both male body size (e.g. Marconato et al. 1989) and nest size (e.g. Lindström 1988, Takahashi et al. 2001) have been shown to positively correlate with the amount of eggs in the nest, in some goby species. In the two-spotted goby, male body size has been shown to influence female mate choice which is biased towards larger males early in the breeding season (Borg et al. 2006). Larger males that are preferred might receive eggs from multiple females and have larger brood sizes than small ones. Temporal variation in environmental factors, such as temperature and food availability, might affect reproductive performance (Donelson et al. 2010) and parental behaviour (e.g. Kvarnemo et al. 1998; Skolbekken and Utne-Palm 2001) and result in variation in the brood size. Thus, male body size, nest size and period were added to the model.

Body size is often positively correlated with territory size in fish species (e.g. Hobbs et al. 2004). The effect of territorial defence on mating success of the neighbouring male might vary according to the size of the male for the same inter-nest distance. Larger males might defend larger territories and more efficiently prevent the establishment of neighbours. Thus, an interaction between distance and male body size (or the absolute differences of the male sizes) in the pairs were allowed in the models.

Results

Occupancy

The probability of double occupancy was not significantly affected by distance. Period did not have a statistically significant effect on the predicted probability of double occupancy. However, the size of the nest had a detectable effect (Table 2), with smaller nests to have higher predicted probability of double occupancy. The predicted probability of double occupancy was higher for the small nests in both periods and at all distances (Fig. 5). For example, at 5 cm distance, early in the breeding season, small nests had 91% predicted probability of double occupancy, as opposed to larger ones that had 69% (Fig. 5). When the interaction between nest size and distance was allowed, the effect of nest size on occupancy was not significant anymore (Table 2). One possible explanation is the loss of statistical power after the introduction of another predictor in the second model, when the interaction between distance and nest size was allowed.

The same models were tested after removal of one location for which only data from July were included. In these models, the variance component for the random effect was estimated as zero, resulting in a singular fit. A singular fit occurs when the number of random effect levels is small or the structure of the random effect is too complex. Another set of less complex models without the mixed factor were therefore tested. The decision of dropping the mixed factor was according to Pasch, Bolker, and Phelps (2013) approach of dealing with singular models where the random variance components are consistently estimated as zero. However, these models are not fully consistent with the experimental design. The parameter estimates for the fixed factor of the singular mixed models and the ones without the random factor were the same. Only the results from the models without the mixed effect are presented (models 3 and 4 in Table 2). Although the interpretation of the parameter estimates should be treated with caution, the results are largely in agreement with the previous analysis that included the extra location. The only notable difference is the weaker effect of nest size (i.e. smaller nests had higher double occupancy than the larger ones, but the difference was smaller). By excluding the data points of this location the number of large nests with single occupancy was reduced, which resulted in slightly smaller difference between single and double occupancy for the large ones ($N_{\text{single}} = 15$, $N_{\text{double}} = 70$ before and $N_{\text{single}} = 12$, $N_{\text{double}} = 68$ after).

Table 2: Summary table of the generalized linear models with binomial error distribution and occupancy as the response variable. All parameter estimates for the different predictors are in logit scale. Distance is log transformed in all the models. Models 1 and 2 are generalized mixed effect models that include location as random factor. Models 3 and 4 are generalized models that do not include location as random factor. Models 1 and 2 include the interaction only between period and distance. Models 2 and 4 include in addition the interaction between nest size and distance.

Predictors	Model 1			Model 2			Model 3			Model 4		
	Estimates	SE	p	Estimates	SE	p	Estimates	SE	p	Estimates	SE	p
Intercept	0.44	0.91	0.626	0.60	0.99	0.547	0.51	0.90	0.574	0.64	1.01	0.528
Distance	0.23	0.23	0.319	0.19	0.26	0.457	0.23	0.23	0.321	0.19	0.26	0.458
Period	1.03	1.38	0.457	1.00	1.37	0.469	1.13	1.52	0.457	1.11	1.52	0.463
Nest size	1.57	0.61	0.01	1.01	1.52	0.508	1.37	0.61	0.024	0.95	1.55	0.543
Distance:Period	-0.15	0.35	0.672	-0.14	0.35	0.689	-0.06	0.40	0.871	-0.06	0.40	0.881
Distance:Nest size				0.16	0.41	0.697				0.12	0.42	0.773
N _{locations}	18			18			17			17		

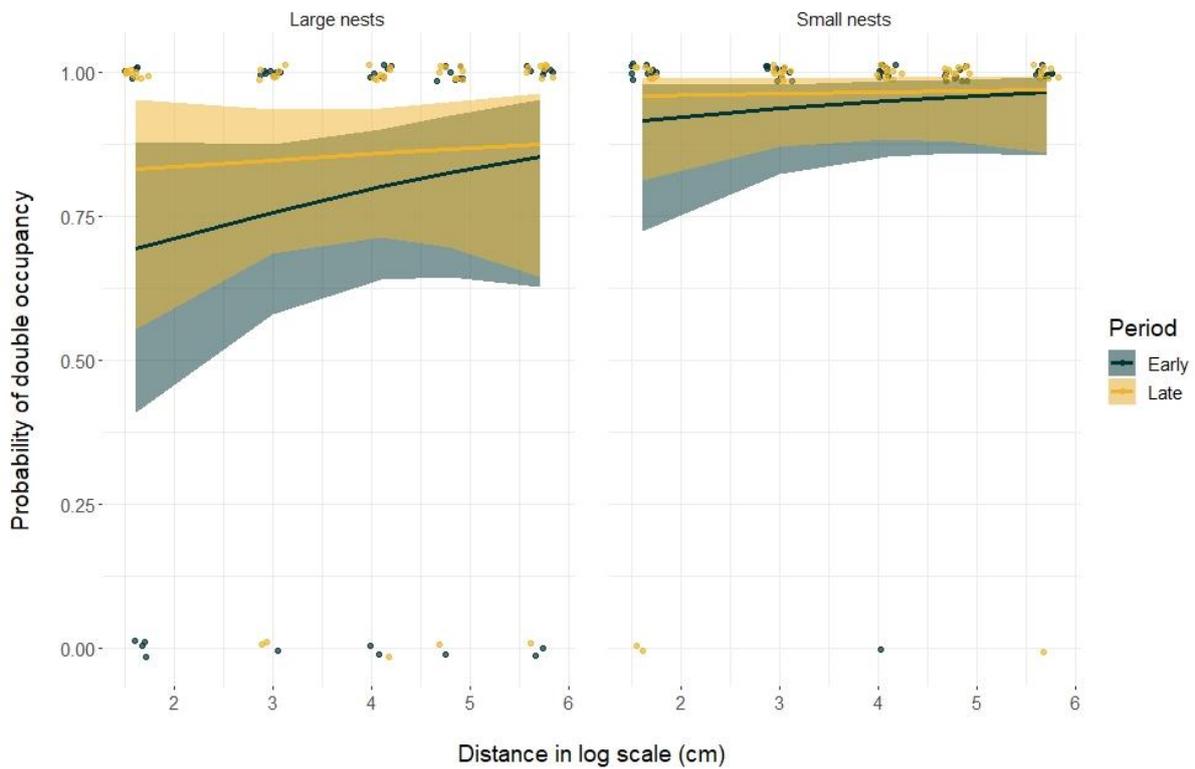


Figure 5: Probability of double occupancy for large (left panel) and small (right panel) nests, as predicted by the first model. The colours correspond to the different periods and the 95% confidence intervals are shown as shaded areas around the predicted responses. The points represent the raw data of observed occupancy in 0-1.

Breeding asynchrony

The probability of asynchrony in breeding was not significantly affected by distance between nests, period or nest size (Table 3). At 5 cm distance, early in the breeding season, small nests had 55% predicted probability of asynchrony in breeding, as opposed to large ones that had 66% (Fig. 6)

The same models were tested after removal of the data points the location with only data from July. The results were similar (Table 3) and thus only the graph from the first model is presented.

Table 3: Summary table of the mixed effect generalized linear models with binomial error distribution and asynchrony in breeding of the two males in the pairs as the response variable. All parameter estimates for the different predictors are in logit scale. Distance is log transformed in all the models. Models 2 and 4 include in addition the interaction between nest size and distance. All models include location as random factor. In models 3 and 4 one location was removed.

<i>Predictors</i>	<i>Model 1</i>			<i>Model 2</i>			<i>Model 3</i>			<i>Model 4</i>		
	Estimates	SE	p									
Intercept	0.75	0.76	0.325	0.46	0.98	0.637	0.73	0.76	0.339	0.41	0.98	0.672
Distance	-0.06	0.17	0.743	0.02	0.24	0.943	-0.06	0.17	0.745	0.02	0.24	0.922
Period	0.06	1.01	0.953	0.12	1.02	0.906	0.01	1.02	0.993	0.06	1.02	0.950
Nest size	-0.46	0.35	0.188	-0.02	1.03	0.987	-0.43	0.35	0.222	0.05	1.03	0.958
Distance:Period	0.14	0.25	0.563	0.13	0.25	0.604	0.15	0.25	0.551	0.14	0.25	0.586
Distance:Nest size				-0.12	0.25	0.644				-0.13	0.25	0.620
<i>N</i> _{locations}	18			18			17			17		

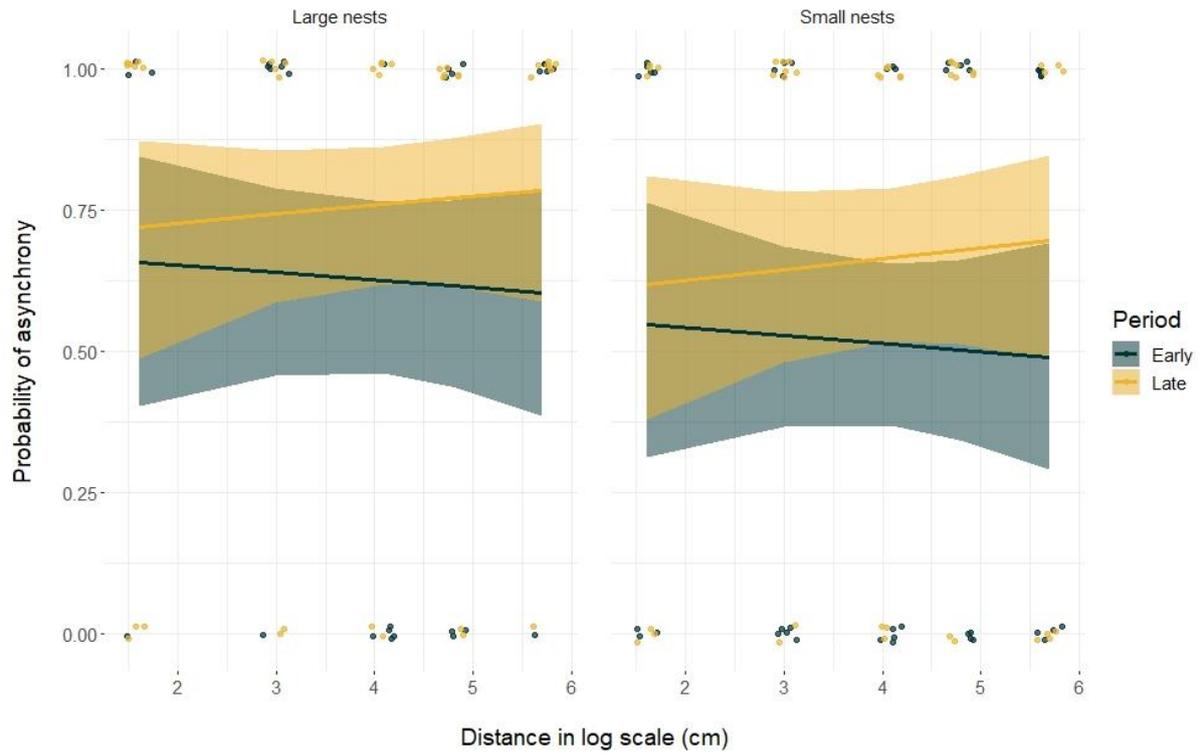


Figure 6: Predicted probability of asynchrony in breeding of the two males in the pairs with distance, for large (left panel) and small (right panel) nests. The different colours correspond to different periods in the breeding season. The 95% confidence intervals are shown as shaded areas around the predicted responses. The raw data in 0-1 are presented as points.

Brood size

No effect of distance or male size was detected on the individual brood size of the males. However, there was a significantly smaller brood size in small nests and later in the breeding season (Fig. 7). The absolute difference between the brood sizes of the two males in the nest pairs was independent of distance, male size difference and period (Table 4). Only nest size had a significant effect on brood size difference of the two males in the pairs, since smaller nests had smaller differences in brood sizes (Fig. 9). The results after the removal of the one location with only data from July are not presented since the parameter estimates were very similar.

Table 4: Summary table of the linear mixed models with individual brood size (model 1) of the males and difference in the absolute brood size of the two males in the pair (model 2) as response variables. Distance is log transformed in both models. Both models include location as a mixed factor. Nest pair (nested within locations) was added as random factor in the first model. The male size difference is in absolute values.

<i>Predictors</i>	<i>Model 1</i>			<i>Model 2</i>		
	Estimates	SE	p	Estimates	SE	p
Intercept	43.12	11.99	< 0.001	7.91	4.32	0.067
Distance	4.23	3.01	0.159	-0.36	0.93	0.701
Male size	4.18	2.44	0.087			
Period	-2.59	0.99	0.009	2.48	1.47	0.091
Nest size	-29.32	1.80	< 0.001	-3.93	1.72	0.022
Distance : Male size	-0.80	0.62	0.0198			
Difference in male size				0.79	5.15	0.877
Distance : Size difference				-0.22	1.26	0.863
N _{locations}	18			18		
N _{nest pairs}	154					

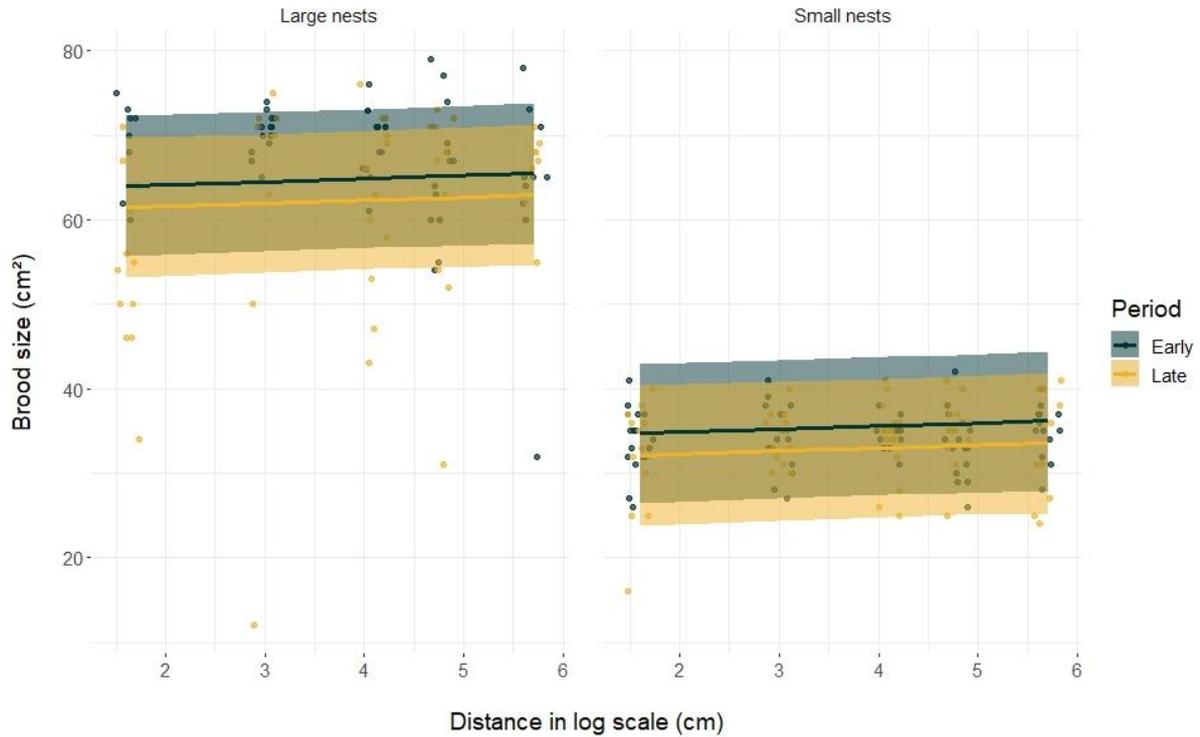


Figure 7: Predicted brood size (measured as number of cm² covered with eggs on the acetates, including hatched and cannibalised areas) with distance for large (left panel) and small (right panel) nests. The colours correspond to the different periods in the breeding season. The 95% confidence intervals (shaded areas) and the observed brood sizes (points) are presented.

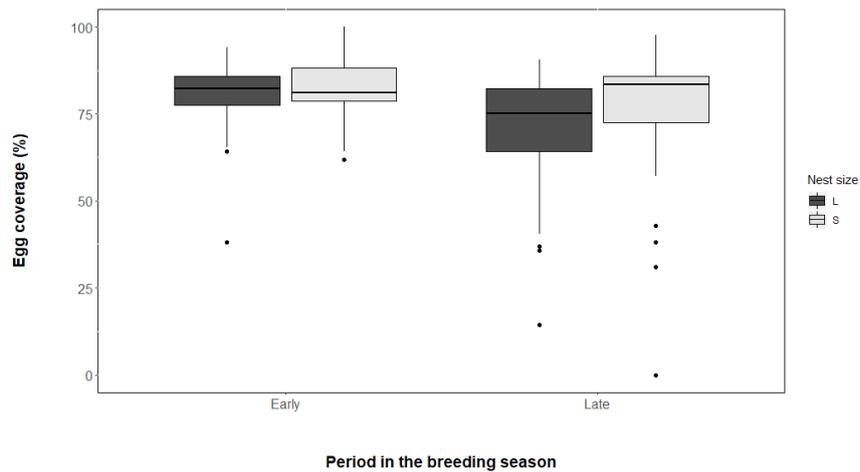


Figure 8: Boxplots with percentages of coverage with eggs (including hatched and cannibalised areas) between early (on the left) and late (on the right) in the breeding season for small (light boxes) and large (dark boxes) nests. The difference in egg coverage (%) between small and large nests was statistically significant for late period, early: mean % coverage for large nests = 80.56, mean % coverage for small nests = 81.36 ($p=0.576$) and late: mean % coverage for large nests = 71.03, mean % coverage for small nests = 77.49 ($p=0.014$)

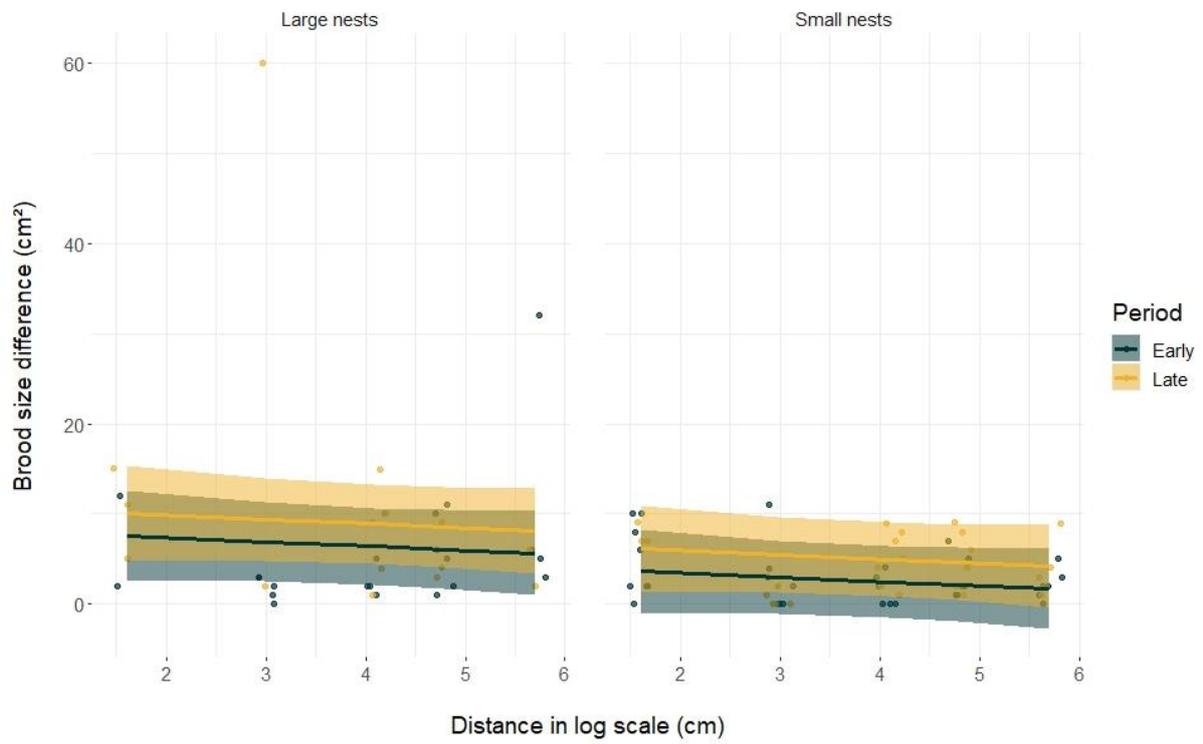


Figure 9: Predicted absolute difference in the brood size of the two males in the pairs with distance for large (left panel) and small (right panel) nests. The colours correspond to the different periods in the breeding season. The 95% confidence intervals (shaded areas) and the raw data (points) are presented.

Discussion

Contrary to my predictions, high occupancy rates were observed for all of the pairs of artificial nests, regardless of distance. With the vast majority of nests occupied by males, no effect of distance was detected. The asynchrony in breeding of males in the pairs of nests was also independent of distance. Likewise, the distance between nests had no detectable effect on either the individual brood size or the absolute brood size difference of the males in the pairs. There was little within breeding season variation in occupancy and asynchrony in breeding rates. However, occupancy was higher in small nests than in large ones. The brood size of the individual males was on average smaller later in the breeding season. The brood size of the individual males was also on average smaller in small nests.

Previous laboratory studies have demonstrated that nest aggregation increases male-male competition and reduces nest occupation rate and mating success, in two-spotted gobies (Mück et al. 2013). Similar patterns were observed in the peacock blenny (*Salaria pavo*) (Saraiva et al. 2009). Under laboratory conditions, the dense spacing of nests promoted nest monopolisation, probably due to the intense intra-sexual competition in the form of territorial behaviour of dominant males, which restricted competitor males from establishing in nearby nests (Mück et al. 2013). Nest aggregation also led to the reduction of brood size, possibly due to trade-off between territoriality and mate attraction and/or courtship interference. Defence of breeding territories or nest sites is also often prioritized over mate attraction in other fish species (e.g. threespine sticklebacks, Candolin 1997; beaugregory damselfish, Santangelo et al. 2002; European bitterlings, Reichard et al. 2004; Smith et al. 2006; zebrafish, Spence and Smith 2005). Competing males might also interrupt each other's courtship and mating, which might affect the overall number of females that males successfully attract for spawning in their nests.

My results contradict these studies since no effects of nest spacing on the density of breeding males and intra- sexual competition was detected. In the field, the shorter inter-nest distances did not seem to promote intra-sexual competition and courtship interference as expected. Shorter distances between the nests did not prevent males from breeding in neighbouring sites or increased the asynchrony in their breeding. Shorter inter-nest distances did not cause a decrease in the brood size as predicted. However, males were observed to engage in competition and aggressive interactions during encounters (pers. obs.). In the field, multiple males simultaneously courted the same group of females and interfered with each other's courtship attempts. During these encounters, males often engaged in aggressive

behaviours towards each other, such as visual displays, chasing and physical attacks (pers. obs.). During the observations in the field, the majority of nest-holders spent a considerable amount of time in their nests and on a few occasions sneaking attempts by other males were recorded (pers. obs.). These observations are in accordance with previous findings indicating that male two-spotted gobies are in fact territorial and engage in intra-sexual competition (e.g. Amundsen and Forsgren 2001; Forsgren et al. 2004; de Jong et al. 2009; Myhre et al. 2012; Wacker et al. 2012; Mück et al. 2013; Amundsen 2018).

In the two-spotted goby, nest defence, provision of parental care and courtship are all energetically demanding activities (Skolbekken and Utne-Palm 2001). Thus, we would expect frequent aggression and courtship interference from close neighbouring males to increase the costs of establishing in adjacent nesting sites. Equally large brood sizes and nest occupancy rates in pairs irrespective of inter-nest distance, suggest that benefits of breeding in the artificial nests possibly override the costs of breeding in close proximity. Previous findings in two-spotted gobies suggest that males suffer accumulated costs of aggression, parental care and courtship effort later in the breeding season (Wacker et al. 2014). The observed reduction of brood and male size later in the breeding season supports this idea. However, whether neighbouring males in pairs with short inter-nest distances suffer higher accumulated costs than owners from nests spread further apart remains unclear. Therefore, observations of the same nest owners are required throughout the whole breeding season. Habituation of the neighbours might also lead to lower levels and therefore lower costs of aggression among neighbouring males. The gradual decrease in aggressive interactions towards neighbouring males was attributed to habituation of the neighbours in male threespine sticklebacks (*Gasterosteus aculeatus*) (Peeke and Veno 1973; Rowland 1988). However, whether habituation occurs in two-spotted gobies remains to be tested.

My results suggest that artificial PVC-nests are likely judged to be high-quality nests by the gobies and the benefits of breeding in such high-quality nests overrides any potential costs of frequent aggression from close neighbour males. The high occupancy of these artificial nests in the presence of natural nesting sites supports that idea. The characteristics of the nest, such as size, architecture and nest openings, are important for the reproductive success of the nest owner in several fish species (Lukas and Orth 1995; Barber et al. 2001; Japoshvili et al. 2012; Lehtonen et al. 2013; Mitchell et al. 2014). The acquisition by the male of a nest with a suitable size or structure influences female choice and his attractiveness to females (Lehtonen et al. 2007; Barber et al. 2001; Mitchell et al. 2014), as well as the risk of egg predation (Lindström and Ranta 1992) and development (Hostache and Mol 1998; Jones

and Reynolds 1999; Takegaki and Nakazono 2000). Female two-spotted gobies have been found to reject males after nest inspections (Myhre 2009, 2012). Thus, the owners of high-quality nests are potentially more attractive to females and enjoy higher spawning success. The shape of the artificial nests with their small nest openings and solid structure makes them difficult to invade by predators and probably offers a high quality substrate for the deposition of the eggs. Attraction to artificial structures has been shown in several fish species (e.g. Lynch Jr. and Johnson 1989) and the use of artificial nests has been very successful in studies of a wide variety of taxa (e.g. Village 1983; de León and Mínguez 2003; Wong et al. 2018), which provides additional support for the preference of artificial nests.

Aggregation of the breeding males may provide some benefits, such as increased female attraction or reduced predation risk, that override the costs of intra-sexual competition. Although in some species males in dense aggregations spent considerable time and energy engaging in territorial aggression (e.g. see Gross, 1980; Sargent, 1981; Unger, 1983), and neighbours often interrupt spawning opportunities (Leiser and Itzkowitz 2003), several studies support the idea that such aggregations are more likely to attract females (e.g. Kynard, 1978; Loiselle, 1978; Gross, 1980; Unger, 1983; Leiser and Itzkowitz 2003) and increase mating success. Breeding aggregation may also offer protection against predators. For example in the bluegill sunfish (*Lepomis macrochirus*), predation of broods has been proposed as a major selective force for the evolution of nesting in dense aggregations (Gross and MacMillan, 1981). Nesting aggregation benefits are less likely to explain my results since we would expect a reverse pattern of occupancy with increasing nest occupancy rates in shorter distances. There are also no studies, to my knowledge, that suggest nest aggregation benefits in two-spotted gobies. However, data of the distribution and density of natural nests is required to fully reject the nest aggregation hypothesis.

High density of breeding males and/or scarcity of natural nests provide additional potential explanations for our results. In cases where the density of males is high or the availability of nests is low, intense competition for the limited nesting sites would increase the costs of defending them. Increased encounter rates as the result of high male density might facilitate competition and monopolisation of matings (Emlen and Oring 1977; Kokko and Rankin 2006) and bias the operational sex ratio (OSR), which depends on the relative densities of the sexes. However, if costs of territorial aggression and competition exceed a threshold, males might lower their level of aggression and allow the establishment of competitors in adjacent areas (e.g. Caballero and Castro-Hdez 2003). For example, in a population of the peacock blenny, nest shortage led to nesting aggregations of males, possibly

due to the strong competition over nest sites and the reduction of aggression between neighbours (Almada et al. 1994). However, it is unlikely that the male two-spotted gobies of the studied population face nest-shortage. Although there was variation among the locations in terms of habitat and population density, the vegetation in the majority of the locations offered a plethora of natural nesting sites (pers. obs.). Since, two-spotted gobies breed in a variety of substrates (Breder and Rosen 1966; Gordon 1983; Forsgren et al. 2004; Mobley et al. 2009; Mück et al. 2012; Wacker et al. 2014; Amundsen 2018) nest shortage is unlikely to explain the observed occupancy patterns. Although potential nesting sites are abundant, high quality nests might be in short supply, which might explain the high occupancy of the artificial nests in the presence of natural spawning sites.

Small nests had higher occupancy rates than the large nests, indicating a male preference for the small nests. The smaller tubes, used as artificial nests, had a narrower diameter of the nest opening than the larger ones, which possibly offers an advantage against predator invasion attempts. Previous studies suggest that male sand gobies build nests that have smaller nest opening size in the presence of a predator (Lissåker and Kvarnemo, 2006). In sand gobies, larger nests with wider openings seem also to be more costly to maintain and defend against egg predators (Kvarnemo 1995) and more susceptible to nest take-overs from rivals (Lindström, 1992).

Smaller nests also accommodated smaller brood sizes. Smaller nests also had smaller differences in the brood size of the two males in the pair. Several previous studies have demonstrated a positive correlation between nest size and brood size, in the sand goby (Lindström, 1988), in a freshwater goby (*Padogobius martensi*) (Marconato et al. 1989), and in the common goby (Magnhagen and Vestergaard 1993). The male preference for smaller nest size as indicated by the higher occupancy despite the limited area implies a trade-off between the costs of maintenance, defence and brood size. The outcome of this trade-off may depend partly on the size of the male, as implied by the difference in male body size between the large and small nests observed in this study. Size-assortative nest choice in fish has been shown by multiple studies (e.g. Lindström 1992; Natsumeda, 2005) also in the absence of direct competition (Kvarnemo 1995). In the small nests, smaller differences in brood sizes were probably due to the fact that the surface area available to accommodate eggs in these nests was easier to fill, as the males would need to attract fewer females to achieve nest fullness. Nest fullness, expressed as percentage of egg coverage, was indeed higher in the small nests than in the large nests, especially later in the breeding season.

The brood size was slightly smaller later than early in the breeding season. Although the effect was marginally significant, it may be due to temporal changes in the condition or the quality of the breeders which might affect mating success and thus brood size. Towards the end of the breeding season both female and male gobies have completed repeated breeding cycles. Given the costs of reproduction and the trade-off between current reproductive effort and both future reproduction (Fisher 1930; Williams 1966) and somatic condition (Williams 1966), a degradation of the breeders' condition may be expected. In support of this idea, studies in a population of the two-spotted goby have shown that nest-holding males were larger early compared to later in the season. Large body size offers a competitive advantage in this species (Wacker et al. 2012) and may be a good indicator of overall male condition. The reduction in nest-holder male size was suggested to be caused by the accumulated costs of mating competition and parental care from the repetitive breeding cycles that prevented larger males from breeding later in the season (Wacker et al. 2014). In the studied population of two-spotted gobies, there was also a seasonal reduction in male size over the course of the breeding season, which was more profound for the large nests that had larger nest owners than the small ones. If body size is an indicator of a general superior competitive ability, the seasonal reduction in male size could explain the reduction in brood size, even if the two measurements are only poorly correlated. Temporal changes within the breeding season in the somatic condition of the males have also been observed in some other fish species, like the peacock blenny (Oliveira et al. 1999) and the mottled sculpin (*Cottus bairdii*) (Downhower et al. 1987).

Conclusion

In conclusion, my results suggest that resource distribution, and specifically inter-nest distance, did not affect intra-sexual competition among male two-spotted gobies of the studied population. The spacing of nests did not appear to limit the density of breeding males and promote nest monopolisation. Limitation of the density of breeding males and nest monopolisation could have skewed mating success and biased the operational sex ratio, with possible implications for the strength of sexual selection. My results suggest that nest spacing and especially inter-nest distance has no apparent implications on intra-sexual competition in this population. However, more studies are required to understand the effect of nest spacing on intra-sexual competition. The results also highlight the importance of nest quality and shortage of high-quality nests in studies of territorial aggression and competition.

The strength of intra-sexual competition, and thus sexual selection, may vary geographically among populations (McLain 1982; Forsgren et al. 1996; Mobley and Jones 2007, 2009; Grant and Grant 2002; Ahnesjö et al. 2008; Siepielski et al. 2009; Miller and Svensson 2014) and according to environmental conditions (Emlen and Oring 1977; Møller 1994). Further studies in other populations of the two-spotted gobies would provide useful insights on the potential geographical variation in the effect of nest spacing on intra-sexual competition. Behavioural observations of same-sex encounters, genetic analysis on broods combined with studies on variation in operational sex ratio, population density and sex ratios would disentangle further aspects of nest competition in the two-spotted goby.

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