Master's thesis

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Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Abstract

Selection of nesting site is an important part of optimizing reproductive success to a lot of oviparous organisms. Choosing the optimal nest may influence multiple life events. In two-spotted gobies, *Pomatoschistus flavescens,* the size of a male's nest is important to the number of eggs he can obtain, the amount of energy he spends on maintenance, the amount of competition he encounters and the male's attractiveness to females. Earlier studies on the two-spotted gobies show that the males often receive enough eggs to fill the natural nests, like mussels, with their maximum capacity for eggs. Therefore, when presented an opportunity to select a larger nest, one might expect the males to prefer this choice. Studies on nest choice in the closely related sand gobies, *Pomatoschistus minus,* have found that the males exhibit size-assortative nest preference in a habitat with a surplus of nest sites and high nest predation, but in an environment without any male competition all males preferred large nests. In my study, I have tested the preference for nest size in a population of two-spotted gobies in West Norway. The habitat is rich in natural nest sites, and two-spotted gobies. The focus of this study was to find out what choice a male would make when it was presented with a small nest, similar in egg-capacity to the mussels, and a large nest with approximately twice the capacity for eggs. The larger nests also come with larger entrances. The results show that there is a preference for small nests early in the season. No preference was evident later in the season. The size of the males is important in explaining the preference of a male. The results suggest that preferences for nest size does exist, and that these preferences change over the season, and with male size. Further studies should quantify male nest choice without females present to avoid female influence. This will isolate male preference with a higher degree of certainty than in the present study.

Sammendrag

Valg av reirplass er en viktig del av å optimere reproduktiv suksess for mange ovipare organismer. Å velge det optimale rede kan påvirke flere livshendelser. Hos tangkutlingene, *Pomatoschistus flavescens*, er størrelse på hannens rede viktig for å avgjøre hvor mange egg han kan skaffe seg, hvor mye energi han bruker på vedlikehold av rede, hvor mye konkurranse han opplever og hvor attraktive han er for damene. Tidligere studier utført på tangkutling viser at hannene ofte får skaffet seg nok egg til å fylle de naturlige redene, som for eksempel tomme skjell, fulle. Derfor ville man kanskje forvente at disse hannene hadde valgt et større rede, om de hadde hatt muligheten til å velge dette. Studier utført på den nært beslektede sandkutlingen, *Pomatoschistus minus*, har vist at i et habitat med overskudd av reir, og høy reir-predasjon, fordelte hannene seg størrelsesfordelt mellom de forskjellige størrelsene på reir. I et habitat uten konkurranse derimot, viste alle hannene en preferanse for store reder. I mitt studie har jeg testet om små eller store reir ble foretrukket i e populasjon av tangkutling på kysten vest i Norge. Habitatet var rikt på naturlige redeplasser og tangkutlinger. Fokuset for denne studien var å finne ut hvilket valg tangkutling-hannene ville ta om de ble gitt et valg mellom et lite rede, på størrelse med et tomt skjell, og et stort rede med kapasitet for om lag dobbelt så mange egg. Disse større redene kommer også med større innganger inn i rede. Resultatene viser at det er en preferanse for små reder tidlig i sesongen. Senere i sesongen er det ingen tydelig preferanse for størrelse. Størrelsen til hannene er viktig for å forklare hva slags størrelse han foretrekker på rede sitt. Resultatene tyde på at det finnes en preferanse i størrelse på rede, og at denne preferansen forandrer seg over sesongen, og med hannstørrelse. For videre studier burde redevalg av hanfisk hos tangkutling kvantifiseres uten påvirkning fra hunfisk. Dette vil isolere hannens valg, slik at vi kan si mer om hva hannen foretrekker.

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Contents

Introduction

Multiple oviparous organisms face an optimization trade-off when selecting egg-laying sites. One important driving force in many of these trade-offs is maximizing one's embryo survival. This might involve both facilitating benefits (e.g. Food availability) and counteract costs (e.g. Predation risk and conspecific disturbances). If this driving force is important, there should be an advantage in fitness for individuals optimizing their choice of egg-laying site (Clark & Shutler, 1999). In many insects, minimizing competition (e.g. Mitchell, 1975; Williams & Gilbert, 1981) and predation (Higashiura, 1989; Petranka & Fakhoury, 1991) seems to be an important driver for selection of egg-laying site. Also, in amphibians, competition and predation has been shown to be an important factor in selection of egg-laying site. For example, some amphibian species tend to avoid waters with predators present (Resetarits & Wilbur, 1989; Vredenburg, 2004), whereas others avoid conspecific presence to minimize cannibalism (Matsushima & Kawata, 2005). The same occurs in reptiles (e.g. Rand & Dugan, 1983) and birds (e.g. Martin, 1992). Avoidance of predation has been suggested to be the most important factor driving nest choice in birds in general (reviewed in Martin, 1993).

Most of these animal groups also have species documented to select egg-laying sites primarily on the basis of optimizing the microclimate for the eggs. For example, in amphibians this might include avoiding egg freezing (Petranka & Petranka, 1981), seeking low salinity habitats (Haramura, 2008) or avoiding areas with high UV-radiation (Palen et al. 2005). In reptiles and birds incubation temperature seems to be highly important (e.g. Schwarzkopf & Brooks, 1987; Davis, 2005) and also important when selecting egg-laying site.

There are alternative hypotheses to what the main driving force behind selection of egg-laying site is (reviewed in Refsnider & Janzen, 2010). Even if there is a lot of data supporting that survival of embryos is important in many species, it has been hypothesized that parental survival is the main driver in some cases. This hypothesis also has support (reviewed in Refsnider & Janzen, 2010). This is not to say that one explanation is opposed to the others existence, but simply that in some species parental survival outweigh embryo survival as the main driver for the selection of egg-laying site. In species, which potentially carry out more than one reproductive cycle during their life, own survival is important to lifetime reproductive success. In cases where one or both parents reproduce multiple times as well as invest time and energy in parental care, these two mentioned hypotheses becomes a trade-off called the fecundity-survival hypothesis (reviewed in Refsnider & Janzen, 2010). In reptiles (e.g. Burger, 1993; Spencer, 2002), insects (e.g. Courtney, 1981; Scheirs, 2000) and birds (reviewed in Ghalambor & Martin, 2001) selection of an egg-laying site based on the survival of the adult has been documented. Every species is not necessarily fixed to one explanation. If there is a conflict, the choice of prioritizing embryo or parental survival may vary with body condition between individuals of the same species (e.g. Amat & Masero, 2004).

In fish, both external fertilization and paternal care often occur (Balshine, 2012). This gives way to a broad range of drivers for selection of egg-laying sites. First, the sperm needs to actually hit and fertilize the eggs when a species has external fertilization. This entails selecting an egg-laying site which promotes this to happen (Petersen et al., 1992). However, the habitat also need to be suited to develop the embryos, and meet the requirements of the spawning adults, as is the case with the other mentioned egg-laying animal groups. Given the vast span of habitats fish use to spawn, a large variety of egg-laying strategies is uncovered. For example, salmonids spawning in rivers have been shown to search for high-temperature sites for egg deposition, to avoid freezing of the embryos (Geist et al. 2002). Other river-dwelling fishes select spawning site to maximize water flow-through, to minimize their energy expenditure on fanning (Bilkovic et al., 2002) or protect against UV-radiation (Probst et al., 2009). In more tropical waters, fish often seem to avoid too hot egg-laying sites (e.g. Middaugh et al., 1981). Several fish species prioritize protection from predators and conspecific cannibalism when they select spawning site (e.g. Middaugh et al., 1981; Spence et al., 2007). In these cases, spacing between nests seems to be important. Depositing eggs on a substrate as opposed to releasing them free-floating in the water column may be an important factor in ensuring protection (Middaugh et al., 1981; Spence et al., 2007). These fishes are referred to as substrate-brooders.

In animals breeding in cavities, for instance birds breeding in nest boxes or many substrate-brooding fishes, the quality of the nest site may significantly affect breeding success (Hoi et al., 1994). For instance, it may be important to have a nest that does not easily allow predators entering to prey on caring adults, eggs or offspring, as predation can be a major source of mortality in the nest (Rand & Dugan, 1983; Lavery, 1991; Martin, 1992; Mobley et al., 2009). At the same time, the cavity should be large enough to contain the maximum number of eggs, or offspring, the caring parent can obtain and care for (Bortolotti, 1994; Lehtonen et al., 2007). For male nest-holding fishes, a small nest can constrain the number of offspring he can produce (Bisazza et al., 1989; Lindström 1992a). Therefore, a large nest constitutes a valuable resource for these males reproductive success. A larger nest may also be more attractive to a female (e.g. Hoi et al., 1996; Lehtonen et al., 2007), adding to the value of the nest as a resource. Even though it seems like males in these cases should have an interest in selecting the highest quality nest they can obtain, it has not been conducted too many studies on male nest choice, but some (e.g. Lindström, 1988; Natsumeda, 1998; Lidström & Pampoulie, 2004; Wong et al., 2008). Wong et al. (2008) have conducted one of these studies on male choosiness. This study described the male choice of nest size in the sand goby (*Pomatoschistus minutus*), a small substrate-brooding fish who dig out nests under cavities, often mussels, in the sand. It was concluded that males prefer larger nests no matter their own size. Other studies on the same species show different results including size-assortative nest preference (Kvarnemo, 1995), and a preference for medium sized nests with a medium sized nest entrance (Japoshvili et al. 2012). The medium sized entrance is constructed probably to avoid nearby sneaker males entering their nest (Svesson & Kvarnemo, 2003), while at the same time allowing water flow-through in the nest (Lissåker et al., 2003). It has been suggested that these different findings can be explained by the differences in the habitats where the studies have been conducted (Wong et al., 2008). For instance, a bigger nest might be better in a habitat where there is little risk of predation or conspecific competition. If these risks are high, however, it might be a better strategy to select the nest you are best able to defend.

Selection for different types of nest architecture has been relatively widely studied in the sand goby (e.g. Lindstöm, 1988; Lindström, 1992b; Kvarnemo, 1995; Lindström, 2001; Svensson & Kvarnemo, 2003; Lehtonen et al., 2007; Lehtonen & Lindström, 2008; Wong et al., 2008; Olsson et al. 2009; Japoshvili et al. 2012). Japoshvili et al. (2012) showed that when presented with a choice between different-sized nests, one large, one small and one medium-sized nest, the males tended to prefer a medium-sized nest, and constructed a medium-sized entrance. This suggests two things. There is a cost to choosing a large cavity size, and entrance size does matter in optimizing nest-site. There might also be other costs of holding a large nest. For example, the cost of fanning increases if a male is relatively small to his nest (Lindström & Hellström, 1993; Künzler & Bakker, 2000). This makes sense, as fanning has been suggested to be more energy costly in large nests containing more water (Kvarnemo, 1995). A wider nest opening may reduce this energy cost for male (Kvarnemo, 1995). Sand gobies dig small holes in the sand leading in under cavities they use as nests like mussels or stones (Lehtonen & Lindström, 2004; Wong et al., 2008) and could probably dig them relatively wide to reduce energy cost of fanning. However, sand goby males in close proximity to other males have been shown to dig out entrances with smaller diameter leading in to their nests (Svensson & Kvarnemo, 2003) suggesting that this behavior in nest building is defensive towards conspecific neighbors. This also underlines the suggestion that these fishes change preference in nest design according to the habitat situation. For organisms such as substrate-brooding fishes and nesting birds, a narrow entrance to the cavity housing the eggs has been hypothesized to prevent larger predators from entering (Martin, 1993; Mobley et al., 2009). Thus, the size of the nest entrance would be beneficial to consider for a male when choosing a nest.

In substrate-brooding fishes, attractive males, and males with attractive nests, may mate with multiple females who deposit their eggs in his nest (e.g. Barber et al., 2001; Mobley et al., 2009; Monroe et al., 2016). Therefore, males may prefer to reside in nests with a large capacity for eggs (Lehtonen et al., 2007). In fishes exhibiting paternal care, male size might be a sign of quality and make a male attractive as larger males have been shown to occupy larger nests and also minimize egg mortality in these nests (Lindström, 1992b; Nelson, 1995; Kvarnemo, 1995; Lehtonen et al., 2007). A possible explanation for this is that the relative size of a nest affect the amount of energy a male has to spend on fanning the eggs. Smaller males will have to use more energy than larger males to support the same nest. This was found to be the case in male sticklebacks (Künzler & Bakker, 2000) and sand gobies (Lindström & Hellström, 1993). Also, Alcock (2000) suggest that a nest, or any other resource, of high quality may attract more competition from conspecifics. Higher quality nests will attract higher quality conspecific competitors (Alcock, 2000) which makes large nests a risk to occupy if you are a small male. In the case of sand gobies, there is a higher chance of having your nest taken over if you are a small male then if you are a large male residing in a nest (Lindström, 1992b; Lindström & Pampoulie, 2004). This might also be the case for other similar species. Often, the size of the entrance is also correlated with the size of the cavity.

This, very likely, is the case when the two-spotted goby (*Pomatoschistus flavescens*) brood in mussels in the wild. The two-spotted goby is a small fish which is semi pelagic and not benthic like the sand goby, but comparable in multiple other ways. They typically use empty mussels (Forsgren et al., 2004; Mobley et al., 2009; Wacker et al., 2014) or cavities in the kelp as nests (Gordon, 1983, Mobley et al., 2009). They also have paternal care (Gordon, 1983), and like the sand goby they defend and maintain the nest throughout the breeding season (Amundsen, 2018). Unlike the sand gobies, two-spotted gobies do not alter their nests (Amundsen, 2018). The natural nests available to male two-spotted gobies vary in both cavity size and entrance size, with the two often correlated. With these variations the male face a trade-off to increase their reproductive success. Often a males spawns with 4-5 females (Amundsen, 2018), at which point a typical mussel often is full (Mobley et al., 2009; Monroe et al., 2016). This implies that with a larger nest they can acquire more eggs while at the same time, possibly, having to both spend more energy fanning, and risking more competition to keep their nest.

In this study, I have quantified the choices made when two-spotted goby males were presented with a choice between a large and a small nest, with large and small entrances. The study was conducted in the field and no data on male occupancy was collected before females appear in the habitat. Hypothetically, the females could be he once deciding which nest she wants to put her eggs in, and then the male starts caring in that nest. It may also be that the nest choices we see are a combination of male and female preference. However, it is quite possible that the males themselves decide which nest they want to occupy, and this study focuses on what size of nest the males occupy. During this thesis, when I mention male choice in nest size, have in mind that this choice may be influenced by female two-spotted gobies. Data has been collected at three different times during the breeding season, as we know there are documented dynamic changes within one breeding season in these systems (e.g. Forsgren et al., 2004; Myhre et al., 2012; Wacker et al., 2014). This leads to the overarching research question: Throughout the breeding season, when presented with a trade-off between a large and a small nest with respectively large and small entrances, what will the male two-spotted gobies choose? It is expected that male two-spotted gobies will show a preference for the large nests, and that the benefits of owning a large nest outweighs any potential costs.

Materials and methods

Study species

Figure 1: Two-spotted goby female (above) and male courting. (Copyright: National Academy of Sciences, photo: Elisabet Forsgren)

Two-spotted gobies are small fishes (35-55 mm total length) (figure 1) (Miller, 1986) that inhabit kelp forests along rocky shores (Collins, 1981; Miller, 1986; Utne-Palm et al., 2015). Formerly the sole species of the *Gobiusculus* genus, the two-spotted goby is now part of the *Pomatoschistus* genus (Thacker et al., 2018) and still part of the Gobiidae family. Both the males and the females are ornamented, still this is a sexually dimorphic species (Amundsen & Forsgren, 2001; Amundsen, 2018). The sexes are distinguishable as the males are characterized by their luminous blue spots which are typically more highlighted in their sex (Amundsen & Forsgren, 2001; Wacker et al., 2013). They also have larger dorsal fins, clearer colors, and recognizable darker spots at both the end of their tail and the root of their pectoral fin (figure 1) (Amundsen & Forsgren, 2001; Wacker et al., 2013). Females are vaguer in their coloration, apart from an orange belly varying in color strength depending on their readiness to mate (figure 1) (Amundsen & Forsgren, 2001) and the carotenoid contents in their eggs (Svensson et al., 2009). The males tend to select female partners with stronger orange coloration revealing their complex sexual dynamic where both sexes are under selection by the opposite sex (Amundsen & Forsgren, 2001). The species is not extensively studied but occur along the coast of Western Europe (Miller, 1986; Patzner et al., 2011). The Nordic populations mainly reside at depths from 0 to 5 m (Amundsen, 2018). They typically use empty mussel shells (e.g. *Mytilus edulis*) (Forsgren et al., 2004; Mobley et al., 2009; Wacker et al., 2014) or cavities in the kelp to breed (Gordon, 1983, Mobley et al., 2009). They only use naturally formed nests, and do not alter or build nests (Amundsen, 2018). Nest-holding males are territorial (Amundsen, 2018) and have been suggested to hold nests half a meter or more apart (Forsgren et al., 2004). They can breed multiple times over the summer season (Johnsen, 1945; Forsgren et al., 2004; de Jong 2011) which lasts from late April or early May, to July (Johnsen, 1945; Forsgren et al., 2004; Wacker et al., 2013). The number of breeding cycles depends on the water temperature (Forsgren et al., 2004). However, they are, mostly, an annual species meaning that most individuals will only have one season to reproduce (Johnsen, 1945). Some fishes survive and live through more than one breeding season (Collins, 1981).

The two-spotted gobies are semi pelagic and leave the kelp only to feed in the nearby water column (Amundsen, 2018). They are often assembled in shoals of 10 or more individuals when not residing in a nest (Amundsen, 2018). When a male two-spotted goby does reside in a nest, it will usually be solitary and territorial (Forsgren et al., 2004). The male will try to court females in proximity to this nest, lead them back to his nest and achieve spawning there. A male can attract several females to spawn in his nest (Mobley et al., 2009). If the male is successful in courting the female she will lay her eggs, 1300 – 1400 on average (Pélabon et al., 2003), in the nest. He will then protect them throughout their development (Amundsen, 2018). Typically, the development will take 1-3 weeks but this depends on the water temperature (Skolbekken & Utne-Palm, 2001; Amundsen, 2018). Parasitic spawning by other males has earlier been reported to rarely happen in this species (Mobley et al., 2009; Wacker et al., 2014) which would make it plausible to assume that eggs belongs to the actual nest-holder. Most of these studies presenting findings on parasitic spawning have been conducted in West Sweden. However, a recent study in West Norway by Monroe et al. (2016) found that up to 30% of nests had been subject to sneaker males, suggesting that this is an alternative reproductive tactic used in this species. These gobies have exclusive paternal care (Gordon, 1983; Skolbekken & Utne-Palm, 2001; Amundsen, 2018) and have never been observed to care for eggs in more than one nest at the time (Mück et al., 2013). Females ready to mate occur both solitarily and in unisexual groups (Amundsen, 2018). They are also competing for males if the ratio between males and females ready to mate is biased towards females (Amundsen & Forsgren 2001; Forsgren et al. 2004; Myhre et al. 2012)

As in other species of fish which exhibit parental care, the two-spotted goby male often cannibalize some of the eggs in his own nest, a behavior called filial cannibalism (Rohwer, 1978). This behavior is likely a way for the male to invest in possible future offspring (Bjelvenmark & Forsgren, 2003).

Study site

The field work of the study was conducted between 30th of April and 8th of July 2018 in the Bispøyan archipelago by Hitra in Trøndelag, Norway (63°32′50″N 8°51′17″E) (figure 2). This work was carried out at five different sub-locations within the Bispøyan archipelago. Steinrenningen (S11) (63°33'46.9"N 8°24'20.3"E), Teistholman (T6) (63°34'44.9"N 8°29'31.2"E), Feøya (F5) (63°35'28.0"N 8°29'49.8"E) and two locations at Vegskiftaholman (V1, V3) (63°33'59.0"N 8°28'37.4"E, 63°34'02.2"N 8°28'07.9"E) (Figure 2).

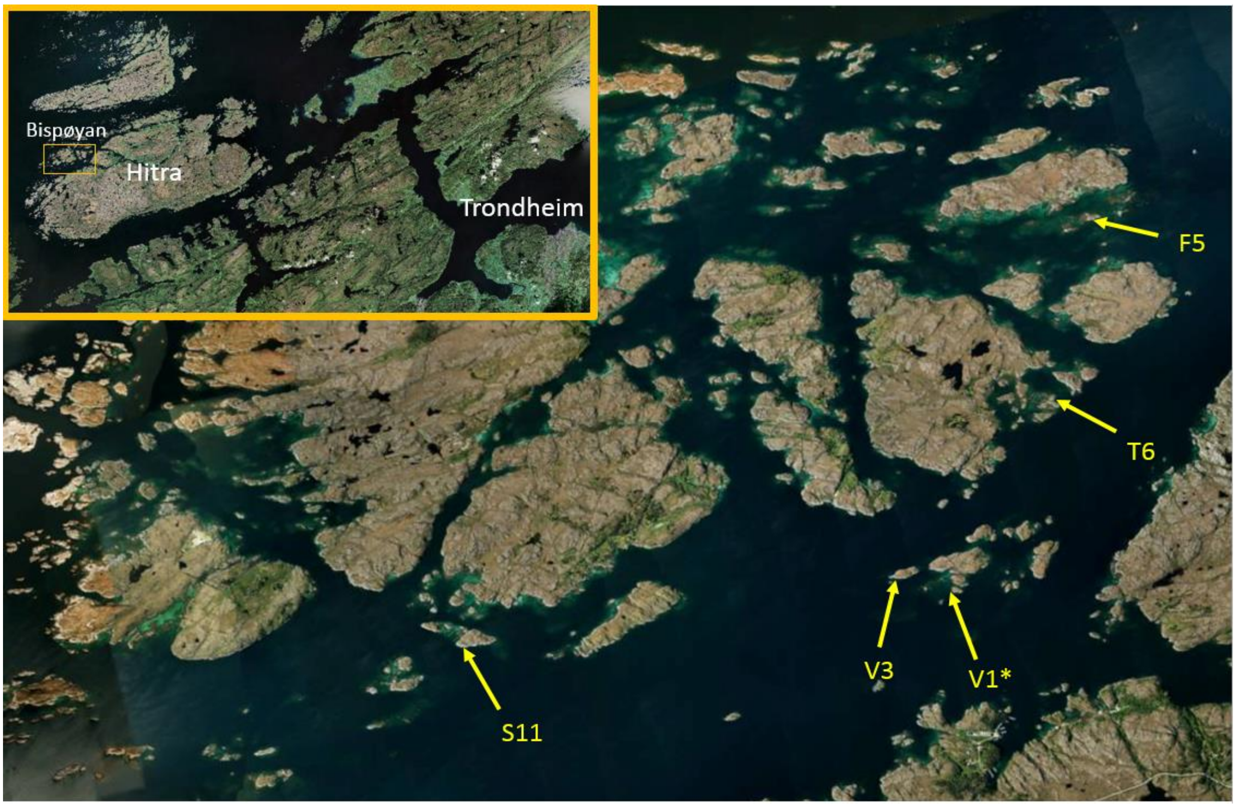

Figure 2: Overview of the study area, the Bispøya archipelago. The samples for this study was obtained from the marked locations; Steinrenningen (S11), Vegskiftaholman (V3 and V1) (* V1 is a location with two strings of nests), Teistholman (T6) and Feøya (F5)

Experimental design

The study was conducted over three different time periods. Nests were placed during a fieldtrip from 30th of April to 6th of May 2018. Clutches of eggs and nest-holding males were collected on fieldtrips at two different times in 2018. First collection of data happened during a fieldtrip from 28th of May to 3rd of June. Second collection happened from 2nd of July to 8th of July. To test male preference between different-sized nests, two-spotted gobies, in their natural habitat, was presented with artificial nesting sites consisting of two different nest types. I introduced one large and one small nest type, with larger and smaller openings respectively (table 1). These two nest types were bundled together (figure 3), which forces the fish to make a choice of which nest to occupy. The nests were PVC tubes, successfully used in previous studies of the species both in the field (Forsgren et al., 2004; Monroe et al., 2016; de Jong, 2011) and in the laboratory (Amundsen & Forsgren, 2001; Skolbekken & Utne-Palm, 2001). The nests make attractive spawning cavities for this species and have previously shown high rate of occupancy. The tubes are inserted with removable acetate sheets to ensure easy collection of egg clutches (Forsgren et al., 2004). These acetates contain a grid comprised of 1 cm^2 squares to simplify quantification of brood size.

One of the tubes was smaller, and had a reduced inner surface (table 1), approximately suitable to host 5 clutches of eggs (Monroe et al., 2016). The other tube offered an inner surface twice the size but had a larger inner diameter and thus opening (table 1). Both tubes had openings in both ends (figure 3). The nest-bundles were connected, by rope, to a brick in one end and a floater in the other (figure 3), and then placed along the shore of the selected locations in the archipelago (figure 2). These locations each hosted a line of nests. Each line consisted of 10 bundles of nests at least 2 meters apart (figure 4). It was deemed more important that each nest was in proximity to kelp or sea weed than that each nest had exactly the same spacing between each other. The minimum of 2 meters between each nest-bundle was used as it was assumed to make the nest-bundles independent, in that the choice in one nest-bundle would not affect the choice in the adjacent nest-bundles. To ensure that the nests were continuously immersed in water they were placed with their floats (figure 3) approximately 50 cm below the lowest tide in the study period.

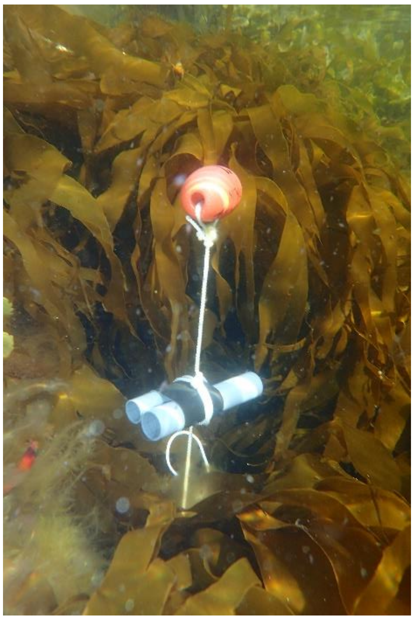

Figure 3: The artificial nesting site as it looks in the two-spotted goby habitat. Consisting of one small and one large tube.

The total number of nest-bundles used was 60 (10x6). Data from all nest-bundles, except the two V1 locations (figure 2), were collected three times (during the last two field trips). At the two V1 locations, data has been collected two times giving the study a total of 160 possible samples if all nests were occupied at all collections. Out of 160 possible samples, 122 were occupied (76 %). Ninety-one nest-bundles were used in the study (see *Photo analysis* for details). Some samples were left out of the study because I found that multiple nest-bundles (65) were, or had been, occupied in both nests (hereafter referred to as double occupancy). This made it necessary to analyze nest contents to determine which nest was occupied first. The samples left out where those nest-bundles where I was not able to categorize it as first occupied in either the small or the large nest. Photo analysis was used to determine what nest was occupied first. After analysis the samples were categorized with labels to describe why a nest-bundle was, or was not, included in the final study. In the 91 nest-bundles used in the study it was possible, with reasonable certainty (see *Photo analysis*), to decide which nest was occupied first. These 91 constituted 75 % of the occupied nest-bundles. Fifty-five of these nest-bundles had a male occupant (60% of nest-bundles used in the analysis) present in the nest determined to be first occupied. These males were caught. Their total length (TL) was recorded and used in the analysis (See *Reasoning for males used in analysis*).

The nest-bundles, during the two first collections, had had a substantial time period to be occupied and filled with eggs. When data were collected from the nests early in the season, the nests had been in the sea 23-33 days. On the next collection, called mid-season in this study, the nests had been out 32-39 days. During the last collection the nests had only had 1-3 days in the water to be reoccupied. Therefore, the last period has less data then the first two. This last collection of data was initially intended as a test to see how fast the fish reoccupied the nests. Their choice was likely made more than a month later then the mid-season group.

Nest dimensions

The small tubes have potential to host approximately 6000 eggs. The most successful males care for more than 10 000 eggs under natural conditions (Amundsen, 2018; Gordon, 1983). The larger tube offers the possibility to double the amount of eggs a male can care for at any given time but it also has a larger entrance diameter (table 1). Therefore, it also comes with the risk of more predation, competition and possibly more energy spent on paternal care.

Table 1: Size of nest-tubes used in the experiment.

Tube type	**Inner diameter (mm)**	**Outer diameter (mm)**	**Inner circumference (mm)**	**length (mm)**	**Area (cm^2)**
Small	16.7	20	52.5	80	42.0
Large	22.3	25	70.1	120	84.1

Location selection and nest placement

Locations were selected before the breeding season based on the presence of suitable habitat for the study species (see *study species*). The nests were placed close to kelp or seaweed beds and, when possible, relatively sheltered from winds and waves. Each location was selected to be statistically independent from other locations in the study. Statistical independence was assumed when there was a body of non-shallow water between each location. At each location the shoreline depth was shallow enough for nests to be accessible for in-water personnel as well as deep enough for each nest to stay fully submerged throughout the breeding season. This means nests were placed at depths from 0.5 to 2 m depending on tide. Nests were placed by snorkelers. Depth of floater from the water surface and distance from neighboring nests were measured.

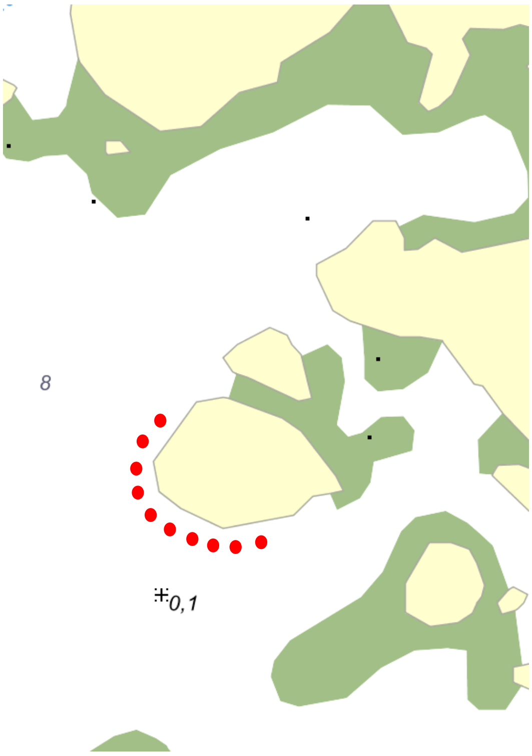

Figure 4: A map of a hypothetical nest location to show how a typical nest line would be placed. The nest-bundles are indicated with red dots along the shore. Each nest-bundle contains both a large and a small nest.

Fish and nest collection

Nests and nest-holding males were collected by snorkelers using handheld nets. To capture the nest-holding male snorkelers used handheld nets or blocked the entrances of the nests with their fingers to trap the fishes inside. The snorkeler observed the nest for approximately 5 minutes before capturing the male (if present) that was determined the nest-holder. A male was assumed to be the nest-holder if he remained in the nest (figure 5), or associated with the nest by displaying behavior like territorial aggression towards other males or courtship leading to the nest. If no male was present in a nest with eggs, the snorkeler waited for 20 minutes for the male to return to the nest, at which point the male was captured and collected together with the eggs. If the male did not return within 20 minutes, the nest with the eggs was collected alone. The snorkeler also collected the nests and brought them to shore. Acetate sheets with egg clutches (figure 5) were collected and replaced before the nests were returned to their original location during the May/June visit. During the July visit, nests were collected and brought on land for the season.

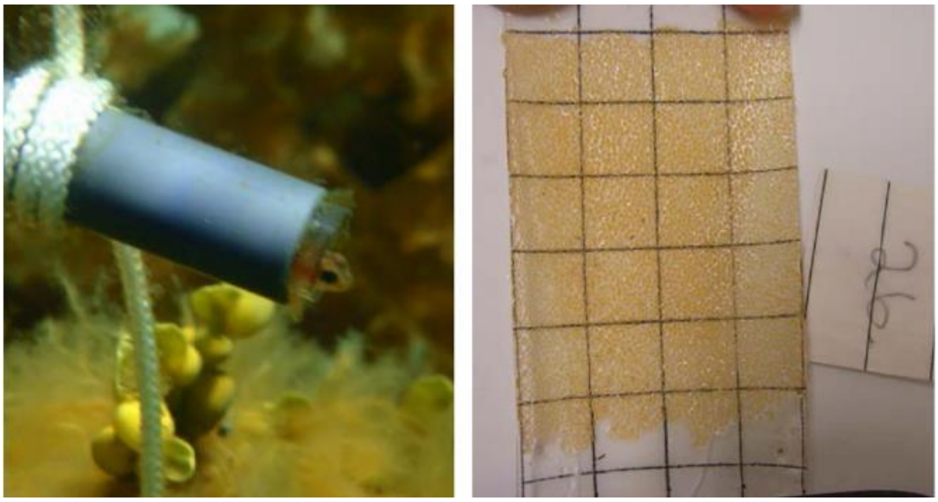

Figure 5: (Left) Photo of a male nest-holder residing in his nest. (Right) Acetate full of eggs after collection. The grid is used for easy brood quantification. The photo is reproduced from previously completed study on two-spotted gobies by Monroe et al. (2016). The materials used look the same as in the present study, with only minor changes in the actual sizes of nests and acetates. Photo: Atikrit Chanjavanakul and Anne Christine Utne-Palm.

Data collection

Nest-holding male TL was measured to the nearest 0.5 mm using a measuring board with a millimeter scale. Each male was killed by an overdose of MS-222 (Tricaine methane sulfonate) and collected. Acetate sheets containing eggs were photographed using a digital camera mounted on a flexible tripod while the acetates were laid flat on a white board as the background, using rubber bands. Both full size photos showing all of the acetate and the whole brood (figure 5), and close-ups of eggs were collected. Hatching of eggs sometimes happened in the container. In these cases a "hatch photo" was taken. A hatch photo is a close-up photo with a transparent background. This makes it possible to record areas previously occupied by eggs, but now hatched. Numbers of squares with eggs were counted and the percentage of the clutch with developed eyes was estimated. Numbers of squares hatched and cannibalized was counted. Areas of hatched eggs have pieces of the eggs still attached to the acetate. This is visible on the hatch photos and differs from cannibalized areas where all of the egg has been consumed. It was important to distinguish between the two as hatched eggs could give valuable information about what nest size was occupied first. Hatched eggs means that a nest has been occupied long enough to at least produce some offspring. Cannibalized eggs does not give any similar information about when a nest was occupied. Therefore, hatched eggs was sometimes used to establish what nest was occupied first if a nest-bundle had double occupancy. All these data were recorded on a standardized sheet.

Photo analysis

All acetates from all nests were analyzed visually to confirm which size nest had been occupied by a male. In cases where only one nest had been occupied this meant confirming that the eggs were present on the acetates belonging to the size of nests recorded on the forms. Sixty-five out of 122 samples had, or had previously had, eggs in both tubes within a nest-bundle. In these cases, all photos were analyzed and the stage of development of the eggs, the area covered with eggs and the presence of hatched clutches was used to determine which size nest had been occupied first, and thereby determining which nest size that was chosen by the first male to establish in a nest-bundle.

The different reasons nests were determined to be first occupied were documented through photo analysis as one of the following: (1) only one nest in a bundle was, or had been, occupied (59 nests). (2) Only one of the nests has a previously hatched brood even if both nests have a brood currently (6 nests). (3) The brood of one of the nests are clearly more developed (15 nests). (4) One nest contained too little eggs to have been established first, or even to be considered established (11 nests). This reasoning was only used when broods had no visual signs of developmental difference.

The nest-bundles which were occupied but not used as samples in the study was not used because of one of the following criteria: (1) both nests were occupied with eggs, but the two broods are deemed too hard to separate into different developmental stages (19 nest-bundles). (2) Both nests have been occupied with eggs, but all eggs are currently hatched making it impossible to analyze the age of the hatched broods (5 nest-bundles). (3) Within- or between person variations in determining what nest was established first in a bundle (6 nest-bundles).

Between- and within-person variation

To assess the reliability of the photo analyses 38 of the nest-bundle’s photos were analyzed, and reanalyzed, by one additional researcher with knowledge on embryo development in *P. flavescens.* 20 of the 38 nest-bundles were chosen at random. The remaining 18 were added because of them being especially hard cases to establish first occupied nest. This additional person was asked to analyze the photos and judge if either the large or the small nest was occupied first, or if they are unable to tell, and thereby inclined to leave that sample out of the study. Out of the 38 samples 17 were used in the final study. In the first analysis, we disagreed on first occupancy in 3 nest-bundles. In the next analysis we disagreed on first occupancy in 2 nest-bundles. Within-person variation was very low in general, and almost non-existing in the subsample used in the study (table 2).

Table 2: Between- and within-person variation. Person 1 (Trygve Norgaard) and 2 (Ioanna Gavriliidi) (P1, P2) have analyzed 38 nest-bundles. This table shows within- and between person agreement in percent in both all nest-bundles analyzed by both persons (n = 38), and the used nest-bundles analyzed by both persons (n = 17).

% Agreement	**Person 1 Within**	**P1 vs P2**	**Person 2 within**
Total (n = 38)	94.7	76.3	86.8
Used samples (n = 17)	100	85.25	94.1

Reasoning for males used in analysis

In the study all males collected in the first established nests were used in the statistical analysis as a fish who has made a choice of which nest to establish in. The assumption is that the male occupying this nest, made the choice to establish in this particular nest as opposed to being forced to establish in a nest because the other nest was already occupied. This is a reasonable assumption for two reasons; (1) Even though, among others, the closely related sand goby are known to have frequent takeovers in the wild (Lindström, 1992b; Lindström & Pampoulie, 2004), there are reason to believe that the two-spotted goby have lower frequencies of takeovers than their close relatives (Mobley et al., 2009; Wacker et al., 2014). Low rates of paternity of any non-nest-holding male in broods indicate that males present in a nest is also likely the owner of the brood in that nest (Mobley et al., 2009; Wacker et al., 2014; Monroe et al., 2016). Therefore, we can be assume that the male holding a nest, is also the male establishing there most of the time. (2) Secondly, if a takeover should have happened, it is not a big problem. A takeover male has still made a choice of what nest size he prefers. For example, if a male choses to take over a large nest where another male lives, he has still made the choice to occupy that large nest.

Data analysis

Statistics

All statistical analysis has been conducted in the statistical software R v.3.5.1 (R Core Team, 2018).

The data collected for TL of fish choosing small and large nests has been tested for normality to determine if a T-test or Wilcoxon rank sum test best fit the distribution. This was done using an F-test to compare two variances. The true ratio of variance in the two groups is not the same (F-test, p = 0.34) which means the Wilcoxon rank sum test is best suited for determining statistical difference between TL in different groups of fish. Quantile-quantile plots also suggest that the data is not normally distributed.

Pearson's chi-squared test was used to test if there was any statistical difference in frequency between the two choices of nest being made. Bar plot of the absolute frequency of large and small nests being selected for each time of season was made by using the package *ggplot2* (Wickham, 2016).

Binomial logistic regression

Binomial logistic regression has been used to model the data as the response has a binary outcome, small or large nest chosen (first). When working on the full 91 samples I used *glmer* and *glm* from the *lme4* (Bates et al., 2015) package in R, a generalized linear mixed model (GLMM) fit by maximum likelihood (Laplace Approximation). The predictors in this case was what time of season the occupation was done, and at which location it was done. Using *glmer*, time of season (early, mid or late) was used as a fixed effect and location was used as a random effect, as there are 6 different locations in the study.

I then used the dataset including length measures on the caught males. This dataset contained 55 samples. When using this dataset I included TL as a fixed effect in addition to time of season. Using the *glmer* function the GLMM model was run with location as a random effect. This resulted in a model with singular fit, likely due to problems in estimating the random predictor variable. Therefore a *glm* was run removing location as a random effect to see the effect on the results. I have used the package *sjPlot* (Lüdecke, 2019) to visualize predictions based on this binomial logistic regression (figure 9).

Results

Occupancy

Among the 91 nests bundles for which occupancy could be analyzed, 51 small nests were occupied first and 40 large nests were occupied first (table 3). The 55 males caught were distributed evenly between nest sizes in total. Twenty-eight were occupying small nests and 27 were occupying large nests.

Table 3: Table showing whether the larger or smaller of two bundled nests was occupied first. Caught males show the number (n) of males collected as first occupants of a nest-bundle of either size S or L. The range and mean of TL is given for the males in each group by mm. Numbers for nests and males are given for three different times of season. Nests established in May, June and July.

Time of season	**Small nests**	**# males (n)**	**Min-max TL (mm)**	**Mean TL (mm)**	**Large nests**	**# males (n)**	**Min-max TL (mm)**	**Mean TL (mm)**	**Collection dates**
Early	26	16	375 - 550	478.4	8	6	450 - 585	508.3	29.05 - 03.06
Mid	19	8	410 - 485	449.4	21	11	430 - 570	500	05.07 - 07.07
Late	6	4	415 - 485	453.8	11	10	420 - 520	450	08.07
Total	51	28	375 - 550	466.6	40	27	420 - 585	483.3	29.05 - 08.07

Throughout the season a number of nests had double occupancy (either a male, eggs or both detected in both nests). Among the 91 nest-bundles used in the study 32 had double occupancy. Most of the double occupancies happened in mid-season when almost half of the nest-bundles had double occupancy (table 4). Even late in the season when nests had only had 1-3 days in the water we experienced 35% double occupancy (table 4). All the nests with double occupancy late in the season had been in the water 3 days, which was the maximum amount of time any late season nest was out.

Table 4: Table showing all occupied nest-bundles throughout the season and how many of these were occupied in both nests. (n = 91).

	Time of season		
	Early	**Mid**	**Late**
Occupied nests bundles	34	40	17
Double occupancy	8	18	6
% Double occupancy	23.5	45.0	35.3

Throughout the season there seems to be a shift in which nests are occupied, or occupied first (figure 7). The odds of a male choosing a small nest in mid-season is significantly lower than the odds of a male choosing a small nest early in the season (table 5, figure 7). Even though there was a relatively high rate of occupancy in the large nests late in the season, it was not significantly different from the choices early in the season (possibly due to small sample size) (table 5). Early in the season small nests are selected significantly more than large nests (table 5).

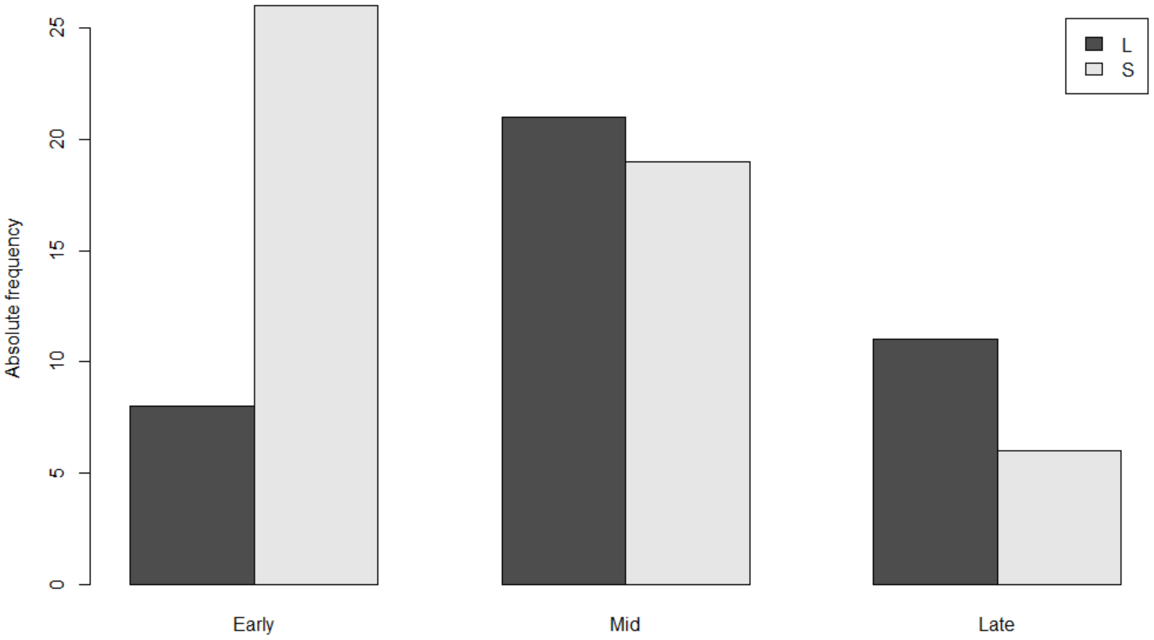

Figure 7: Absolute frequency of nest sizes occupied first in a nest-bundle at different times during the breeding season. Time of season is defined by when the nest was occupied, not collected. (n = 91).

Table 5: Summary table of the GLMM with time of season as the predictor and nest size as the response variable. Parameters are estimated in logit scale. Model 1 includes locations as a random effect. Number of stars (*) signifies to what significance level the predictor I significant. (n = 91).

	Model 1		
Predictors	**Estimates**	**SE**	**p**
Early	1.426	0.618	0.021*
Mid vs early	-1.545	0.586	0.008**
Late vs early	-1.204	0.721	0.095

Effect of total length of fish on nest choice

The distribution of nests occupied first which also have data on male TL (n = 55) is similar to the distribution when all data is included (n = 91). The preferred nest choice is still small early in the season, but changes to large nests late in the season. In mid-season the preferred nest size is more evenly distributed. There is no statistical difference in the males TL choosing small and large nests in early or late season, but in mid-season there is a significant difference (Wilcoxon rank sum test, p = 0.016), where larger males tend to occupy larger nests and smaller males occupy smaller nests. Also, the nest-holders, over

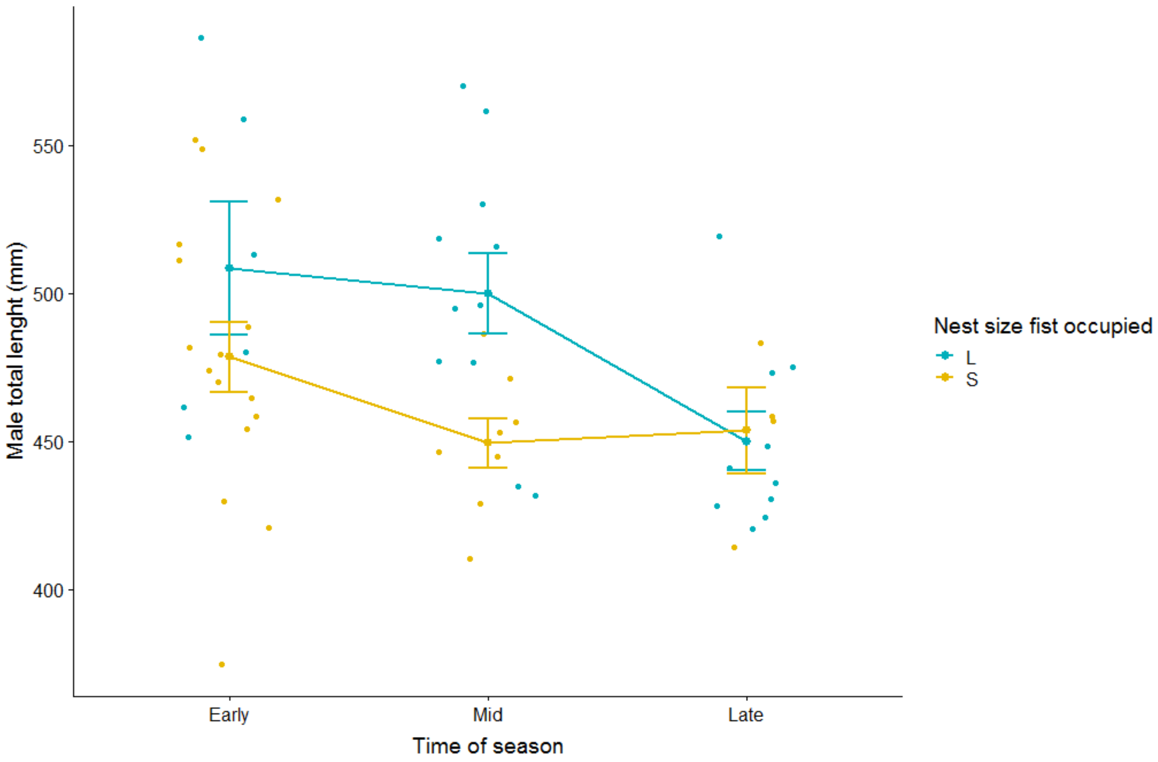

Figure 8: Total length of fish occupying large and small nests throughout three different times of the breeding season; Early, Mid and Late. Time of season is defined by when the nest was occupied, not collected. (n = 55).

all, are significantly larger early in the season compared with late in the season (Wilcoxon rank sum test, p = 0.015) (table 3, figure 8).

The glmm and glm models (model 2, model 3, table 6) show that all times of season and the TL of the male is significant in explaining the male nest choice when TL is included as a fixed effect. The mixed model including location as a random effect could not be fully estimated due to singularity problems and a regular logistic regression without the random effect was run to confirm the effect of the factors. The models show that the choice of nest size is significantly affected by time of season. Early in the season the probability of choosing small nests is generally higher than random but later in the season the probability of choosing small nests is reduced (table 6, figure 9). In addition to seasonality we see that the choice is also affected by the TL of a male (table 6, figure 9). The results show that the odds of a male choosing a small nest significantly changes as a males' body length changes. If a male is large, the odds of him selecting a small nest drops. The smallest males early in the season are predicted close to a 100% chance of selecting a small nest (figure 9). Compared to late in the season, the smallest males are predicted less than 65% chance of making the same choice (figure 9).

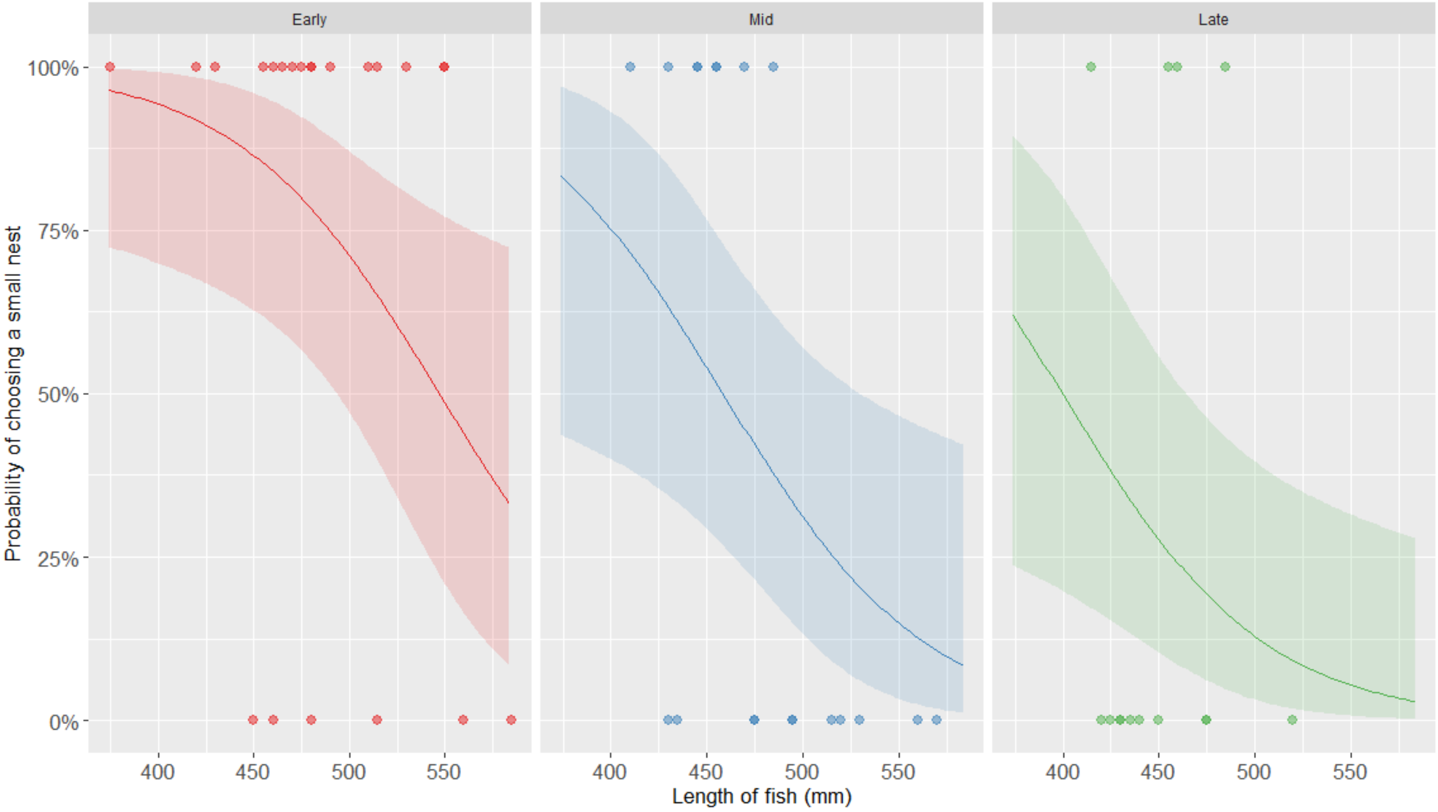

Figure 9: Graphs showing the relationship between total length (TL) of a male along x-axis, and the probability of that male of occupying a small nest along the y-axis. The three different colored curves, red, blue and green, indicates the choice probability early, mid and late in the season respectively. (n = 55).

Table 6: Summary table of the GLMM with time of season and TL (total length) as predictors and nest size as the response variable. Parameters are estimated in logit scale. Model 2 includes locations as a random effect. Model 3 does not include data on locations. (n = 55).

	Model 2			*Model 3*		
Predictors	**Estimates**	**SE**	**p**	**Estimates**	**SE**	**p**
Early	10.402	4.153	0.012*	10.402	4.200	0.013*
Mid vs early	-1.690	0.762	0.027*	-1.690	0.763	0.027*
Late vs early	-2.808	0.928	0.002**	-2.808	0.931	0.003**
TL	-0.019	0.008	0.020*	-0.019	0.008	0.022*

Discussion

Nest occupancy

122 of all the 160 nest-bundles used were occupied (76%). Out of these 122 nest-bundles, 65 had double occupancy (53%). Compared to early in the season, the occupancy is lower late in the season when only 42.5% of the nests are occupied. During early and mid-season the occupancy rate is 87.5%. The artificial nests have a high occupancy rate, and are probably popular nests compared to a lot of the natural nests. These nest have also been successful in previous work in the field (Forsgren et al., 2004; Monroe et al., 2016; de Jong, 2011). The low occupancy rate late in the season might be because of a decline in male abundancy. This drop in male abundance has been suggested before (Forsgren et al., 2004). It is also possible that the occupancy rate in late-season is lower because of the short time period these nests had in the water. I suggest a combination of the two as the occupancy rate is, relative to earlier in the season, very low late in the season.

It was not expected that a nest-bundles would have double occupancy. In two-spotted gobies, activities such as nest defense and courtship are energy costly (Skolbekken & Utne-Palm, 2001). Therefore, it was expected that two males would establish in nests further apart to avoid aggression and competition. The high rate of double occupancy suggests that the artificial nests provide benefits which outweigh the potential costs of having a neighbor just a few centimeters away. The popularity of these nests may have influenced the results. For example, it is possible that the quality of both the small and the large nest were so good that the males inhabiting them experience more competition from other males than they would in a natural nest. If there is a lot of competition around your nest, and the potential for another male to occupy a nest up close to yours is high, then maybe the benefit of having a small entrance to your nest outweighs the benefit of having a large nest. In sand gobies males have been shown to dig out smaller entrances to their nests when they reside in close proximity to another male (Svensson & Kvarnemo, 2003).

Nest size preference (n = 91)

There was a change in preference over the breeding season. Early in the season small nests were preferred over large nests, whereas no such preference was evident later in the season. The change in preferred nest size that happens from early to mid-season is significant. There are well documented changes in the sexual dynamic within one season in these systems, including changes in OSR (operational sex ratio) (Forsgren et al., 2004), direction of sexual selection (Myhre et al., 2012) and the total length of the nest occupying males (Wacker et al., 2014). In sticklebacks it has also been found that smaller males arrive earlier to the breeding grounds (Candolin & Voight, 2003). Therefore, the preference changing throughout the season was not very surprising, even though it was not expected. It has been suggested that the abundance of male two-spotted gobies significantly decline throughout the season (Forsgren et al., 2004) at the same time as the size of the nest-holding males decline (Wacker et al., 2014). In this study, the occupancy rate declined towards the end of the season. This might suggest less competition over the nests later in the season.

As we enter mid-season, the odds of a male choosing a small nest significantly decreases compared with early in the season. This happens without regard for male size. This suggests that there might have been a seasonal benefit to breeding in small nests which is now gone.

I suggest a potential explanation linked to the fecundity-survival hypothesis. We know that the two-spotted goby produces multiple broods during one season, if able to (Johnsen, 1945; Forsgren et al., 2004; de Jong 2011). The male also provide care for each brood (Amundsen, 2018). Providing care, nest defense and at the same time court females is a very demanding task for these fish, and is energetically costly (Skollebekken & Utne-palm, 2001). Larger nests may also be subject to both predation (Kvarnemo, 1995) and take-overs (Lindström, 1992) more frequently than smaller nests. The fecundity-survival hypothesis suggests that for a parent in this situation there are two major drivers influencing the selection of a nest (Ghalambor & Martin, 2001; reviewed in Refsnider & Janzen, 2010). These drivers are the survival of the parent, and the maximum survival of embryos. Wacker et al. (2014) suggested that males suffer the consequences of early excessive energy use later in the breading season. It might be the case that the risk of takeovers and competition from high quality males is too high to risk in the large nests, and pose a threat to the male's survival. Competition can lead to higher mortality (Lindström, 2001). Also, the care of more eggs in the large nest could be exhausting (Marconato et al., 1993; Skollebekken & Utne-palm, 2001). I suggest that the drive for parental survival is higher earlier in the season as the potential of spawning more broods later in the season is higher. This could explain why even males larger than the population mean are more likely to choose small nests early in the season.

A separate explanation to what we see might be similar to what Svensson and Kvarnemo (2003) found in a population of sand gobies. Since there seems to be a very high occupancy in early and mid-season, it could be that the male's lives in such close proximity to each other that they choose small entrances over large cavities, because this gives the bigger benefit. A smaller entrance might help avoid sneaker males entering the nest. It may also help you avoid another male living in a nearby nest (e.g. right beside you) entering ad cannibalizing your eggs. If we consider the amount of double occupancy, this makes even more sense. However, the males do not tent to choose small nests in mid-season, and at this time there is also a very high occupancy, and even higher rate of double occupancy compared to early in the season (table 4).

Nest size preference regarding fish size (n = 55)

I found that the size of the fish is significant in explaining change in nest size preference. When male size is accounted for, the change in preference from early to both mid- and late-season is significant. Early in the season the size of male nest-holders is significantly larger compared to late in the season. I also found that the size of the large nest-holders is significantly larger than the size of the small nest-holders in mid-season.

We know that the size of the nest-holding male two-spotted goby has been found to be larger than the population mean early in the season (Wacker et al., 2014). The mean size of male nest-holders in this study was significantly lower late in the season than early in the season. It would be reasonable to expect that nest size preference would be gradually skewed more towards small nests if male nest-holder size decreased through the season. Actually, as size decreases the males go from selecting small nests to having no preference in nest size later in the season. However, the change in choice that happen from one time of season to another is significant. This change evens out the odds of a male choosing a large or a small nest. This might happen because of a higher male mortality due to competition earlier in the season (Lindström, 2001), or even the energy cost of parental care (Marconato et al., 1993) that some of the males later in the season might have accumulated (Wacker et al., 2014). This study suggest that there are less males competing over nests late in the season. This has been reported by others to be the case over the season in this species (Forsgren et al., 2004; Myhre et al., 2012). It may be the case that smaller males occupy larger nests later in the season which earlier in the season was unavailable due to competition from more and larger males. With some of the high quality males out of the competition, and an occupancy rate decreasing to 42.5%, it might be that the larger nests are less risky to occupy as nest take-overs might be less likely for an average sized male.

It may also be the case that the size of the nest is more important than size of the entrance of the nest late in the season. Maybe because there is a lower rate of occupancy late in the season (42.5%), and hence less risk of sneaker males. Ten the opportunity to mate with more females could outweigh the risk of sneaker males and cannibalization from conspecific males.

Size assortative nest choice

Mid-season is the only period where it looks like we have a pattern of size assortative nest choice (figure 8). This is not a new phenomenon as studies have found this pattern in other fish species (Lindström, 1992b; Kvarnemo, 1995; Natsumeda, 1998). My models show that changes in male size significantly changes the odds of a male choosing one or the other nest size (figure 9), and this is very clear in mid-season where the nest holders of large nests are significantly larger than the nest holders of small nests (figure 8). As opposed to early in the season when all males under the total length of 550 mm had a higher chance of selecting a small nest than a large one, in mid-season males of approximately 460 mm and above have a higher chance on choosing a large nest than a small.

Conclusion

In conclusion my results show that males choose smaller nests over large ones early, but not late in the season. This is a clear, and significant. The results tells us that the progress of the season significantly alters the male’s preference. Although there is a change, these data do not imply that any nest size is significantly more popular than the other late in the season. The data merely indicates a tendency of nest preference being skewed from small nests clearly being preferred, towards large nests being more preferred.

The results also show that the size of a male significantly alters his odds of choosing one or the other nest size. A male is gradually less likely to choose a large nest the smaller he is. For example, early in the season, a male with the total length of 550 mm will have approximately 50% chance of choosing a large nest. However, a male with the total length of 375 mm will only have 2-3% chance of choosing a large nest at the same time of season. This most clearly presents itself in mid-season where the males seem to size assort themselves between the two sizes of nests. Later in the season male nest-holders are significantly smaller than early in the season, but still they are significantly more likely to choose a large nest compared to early in the season.

I suggest that there is a trade-off between fecundity and survival, and that this trade-off might be, at least, part of the explanation of the pattern this study has found. The costs associated with occupying, maintaining and defending a large nest may be too great for a male at the start of the season to risk suffer the accumulated costs later in the season. At this time more males may chose small nests to save energy, and invest that energy later in the season. By the end of the season there is, for most males, less of a benefit in investing in the future, and therefore investing in present fecundity is the best option. In a new dynamic, with a female biased OSR and less conspecific male competition, the larger nest is now a good option to produce as many offspring as they can before the breeding season ends.

The high rates of occupancy in the artificial nests, and the surprisingly high rates of double occupancy, imply that these nests are considered high quality. I suggest that the high occupancy rate might alter the choice made by male two-spotted gobies. In a densely populated area it might be more beneficial to have small entrances to your nest, than to have a large nest with large entrances. A small entrance might avoid sneaking, or cannibalization, from males in close proximity.

Future research should isolate male two-spotted gobies and present them with a nest choice. If this can be done, we would understand more about male preference in nest size as we could rule out the possibility of females being part of the choice. Also, if competition is controlled, we can see male’s preference uninterrupted. I also suggest that future field studies extend research into late July, to be able to get a full understanding of how nest preference changes over the season. The last sampling in this study was both smaller and had little time under water. The finding are however interesting, and third sampling should be done later in July with as many samples at the previous samplings.

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