The Role of the Novel Female Ornament in the Wide-bodied

Pipefish, *Stigmatapora nigra*

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School of Biological Sciences

University of Canterbury

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Abstract

Sexual selection can drive the evolution of exaggerated ornaments and armaments, which serve no purpose to survival but are beneficial for mate choice or intrasexual competition. Sexual selection theory was based on elaborate sexual traits displayed by males, with the expectation that males invest less in gametes, as sperm is less costly than eggs, and thus freeing up males to invest in sexually selected traits. Despite several taxa expressing female ornaments, there have been underlying assumptions that ornaments will have drastic fitness costs for females as a result of trade-offs with fecundity. This raises the question of whether female ornaments can evolve through sexual selection, and act as honest signals during mate choice or evolving through correlated inheritance with male traits.

In sex-role reversal, sexual selection acts more strongly on the female sex, and females compete for mating opportunities, which can lead to the evolution of ornaments. Sex-role reversed species are therefore good model systems to investigate sexual selection on female ornaments, and whether sexual traits can exist without requiring trade-offs with reproductive investment.

Stigmatapora nigra, the wide-bodied pipefish, is a sex-role reversed species in which the female displays an elaborate ornament. Under sex-role reversal, I would expect the female to be the courter, hence initiating courtship, whilst the male would be the chooser, and thus selective in mate choice. Little is known about the role of this novel female ornament; whether it is used in mate choice and/or female-female competition, and whether it honestly signals female quality. This formed the basis of this research.

Through mesocosm experiments I observed the courtship displays of *S. nigra* and found that the female ornament was actively displayed during courtship. Most of the female displays observed were directed towards males but occasionally females were seen directing displays towards other females, which may be an indication of female-female competition. Surprisingly, males initiated more courtship events than females and there was

no difference in the proportion of active courtship displayed by both sexes, which was contrary to my predictions.

To investigate potential trade-offs between female ornaments and fecundity, I dissected females from three sites around New Zealand. Larger, more ornamented females were predicted to be larger, heavier, and have more eggs. Females maintained honest ornaments across all populations, despite variation in ornamentation and body size existing between sites. Overall, the ornament of *S. nigra* was predominantly displayed during courtship and reflects the reproductive quality of the female, suggesting that it may be important in male mate choice and thus likely to be under sexual selection.

Sexual selection can therefore explain the evolution of some female ornaments, and previous assumptions regarding trade-offs do not stand for female *S. nigra*. Some of the courtship behaviours of *S. nigra* were surprising, and potentially related to the fact that sexual selection can be dynamic and mating behaviours can fluctuate depending on the social environment. As this field of research progresses there should be more emphasis on sexual selection in both sexes and the plasticity of these selection pressures across space and time.

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Chapter 1: The Evolution of Female Ornaments

Sexual selection drives the evolution of extreme traits, such as ornaments and armaments (Darwin, 1871). Ornaments are elaborate signal traits that bear some cost to the carrier and serve no purpose to survival but provide a benefit in terms of reproductive success (Kraaijeveld, 2019; Kraaijeveld et al., 2007). Across the animal kingdom, species display ornamentation or forms of sexual dimorphism. Examples include the elaborate and well-known ornamental tails of peacocks *(Pavo cristatus)* (Loyau et al., 2005), iridescent blue lateral spots in male two-spotted goby (*Gobiusculus flavescens*) (Amundsen, 2018), the extremely elongated rostrum in male New Zealand giraffe weevils (*Lasiorhynchus barbicornis)* (Painting et al., 2014; Painting & Holwell, 2014), and colouration in Iberian green lizards (*Lacerta schreiberi*) (Megía-Palma et al., 2018). Although male ornaments are more commonly found in species, female ornaments are still expressed in a wide range of taxa. However, historically they have received little attention (Amundsen, 2000a). A wide range of female ornament expression exists, from dull to elaborate colouration. In some species, females are more highly ornamented than males (Berglund & Rosenqvist, 2001; Bussiére et al., 2008; Flanagan et al., 2014; Owens et al., 1994). How and why females have evolved to express these striking traits is still under question, with several explanations and pressures proposed and investigated. This knowledge gap forms the motivation for this study. The alternative hypotheses for the evolution of female ornamentation will be explored in this review, along with the assumptions of female ornament signalling and constraints, highlighting gaps in research into female trait evolution.

Evolution through correlated inheritance

Darwin proposed that female ornaments were non-adaptive, a result of sexual selection acting on the males, leading to vestigial expression of male ornaments in females (Amundsen, 2000a; Darwin, 1871). Lande (1980) showed that ornaments displayed in both sexes were not independently inherited, as genetic models suggested that selection on males resulted in a correlated response in the female sex (Amundsen, 2000a; Lande, 1980).

Sexual dimorphism was thought to arise through a very slow process of evolution, causing females to lose the male ornament over time (Lande, 1980). Lande (1980) proposed that female ornaments which appeared as a diluted version of the male ornament were still in this evolutionary process, working towards complete sexual dimorphism (Amundsen, 2000a; Lande, 1980). Correlated inheritance assumes that only male ornaments can be under sexual selection and that female ornaments are non-adaptive, ignoring the pressures of mutual mate choice (Amundsen, 2000a; Tobias et al., 2012).

In the case of mutual ornamentation, female ornaments have evolved in part through genetic correlation (review in Kraaijeveld et al., 2007). For example, in the Zebra finch red bill colour is genetically correlated between the male and female sex. A cross-fostering aviary experiment by Price (1996) demonstrated that the same genes were causing an effect on bill colour expression in both males and females. Red bill colouration was selected for in males and selected against in females, through genetic correlation, genetic load was formed resulting in neither sex evolving towards their selective optima for bill colour (Price, 1996). More recently a study examined the genetic correlation between female and male tail ornaments in a number of hummingbird species (Clark & Rankin, 2020). When males expressed ornamental tail traits, which are used during courtship to create sounds, subtle tail traits were also present in females (Clark & Rankin, 2020). The subtle ornamental male traits present in females have no adaptive function and female tail shape was not under direct selection, as no sound is created. Female tail shape has thus evolved due to genetic correlation between the sexes (Clark & Rankin, 2020; Robert, 2020). However, another recent study found the contrary, in which female-limited polymorphism, when females express male colouration, was not explained by genetic correlation throughout the hummingbird lineage (Diamant et al., 2021). Instead, preliminary support was found for social selection pressures acting on these female ornaments, indicating that it may be adaptive for females to display male-like colouration (Diamant et al., 2021). Similarly, in the case of sexual dimorphism, when females maintain ornaments different to those displayed by males, other factors besides correlated inheritance, such as mate choice and competition, have likely contributed to female ornament evolution (Doutrelant et al., 2020).

Evolution through competition

Competition is a selective force driving the evolution of some female ornaments. Females can evolve ornaments beneficial for ecological resource competition (i.e., food), access to mates, or high-quality resources for reproduction (Clutton-Brock, 2009; Doutrelant et al., 2020). Thus, female ornaments can evolve in both sexual and non-sexual competitive contexts (Amundsen, 2000a, 2000b; Hare & Simmons, 2019; Tobias et al., 2012). These ornamental traits signal the competitive ability of the individual without the need for a costly physical fight (Midamegbe et al., 2011). Larger, brighter ornaments correlate with competitive ability, providing information to rivals on the dominance status of the bearer (Berglund & Rosenqvist, 2008; Earl et al., 2021; Leitão et al., 2019). This form of signalling has been well studied in male-male competition and is also found to contribute to female ornament evolution, although the costs and benefits of expressing competitive traits may differ between the sexes (Cain & Rosvall, 2014).

Competition for territory and resources

Competition for social and ecological resources can act as a key selection pressure, favouring the expression of female ornaments (Macedo et al., 2021; Thys et al., 2020). For example, in the pied flycatcher (*Ficedula hypoleuca*) some females maintain a white forehead patch, which is used in intrasexual interactions to signal dominant status (Morales et al., 2014). Territory intrusion simulation experiments found that females were more likely to attack non-patched decoys over patched ones, with ornamented females carrying out these attacks more frequently than non-ornamented females (Morales et al., 2014). Another study on pied flycatchers found that females bearing a white wing patch avoided intrasexual competition for nest cavities, decreasing the need for vigilance and allowing better incubation attendance (Plaza et al., 2018). Female white shoulder fairywrens, from

the subspecies *Malurus alboscapulatus moretoni,* who had shorter tails (a sign of dominance), were found to be more aggressive and more coordinated with their mating partner, indicating that ornamentation had evolved along with other signals as a response to increased social selection pressures (Jones et al., 2021). In the cichlid fish *Tropheus*, females with a wider yellow bar ornament were advantageous in winning size-matched female-female competitions for territory (Ziegelbecker et al., 2018). Hence female ornaments in the form of sexual monomorphism may arise to show competitive abilities during territory possession (Ziegelbecker et al., 2018).

Female ornaments can also be used as status badges for territories outside the breeding season, as shown in the hummingbird species, *Panterpe insignis* and *Eulanpis jugularis,* where females have bright monochromatic plumage, used during food competition (Wolf, 1969, 1975). Females that defended feeding territories displayed bright plumage as a signal of dominance, in contrast, females from non-territory holding species were dull with no ornamentation (Wolf, 1969, 1975). Status badges are also used during intrasexual competition, for vital resources essential to reproduction. In the dung beetle species, *Onthophagus sagittarius,* females maintain a horn which is used in female-female competition for dung, an important resource for reproductive fitness (Watson & Simmons, 2010). The size of the female horn predicts the outcome of the competition, with large horned females having the advantage, winning more female-female battles (Watson & Simmons, 2010). By displaying honest status badges, costly and potentially harmful competition events can be avoided, as subdominant females will avoid initiating fights with more dominant females.

Intrasexual competition for access to mates

In several cases, females compete with other females for access to mates, another form of competition that can drive the evolution of female ornamentation. Intrasexual competition for mates may arise if there are benefits to the female, such as gaining access to high-quality males, securing a mate when territory-holding males are limited, or competition for male parental care (Amundsen, 2000b; Rosvall, 2011). Murray et al. (2020)

found that heightened competition for access to mates drives ornamentation, with dance fly species showing greater female ornamentation when lekking swarms had a female-biased operational sex ratio, hence intrasexual competition was elevated. In Eurasian dotterels, females are the more ornamented sex and compete in mating arenas for access to males (Owens et al., 1994). Brighter females were found to court more frequently, as well as initiate and win more of their fights (Owens et al., 1994). Bright plumage acts as a deterrent, as dull individuals will not initiate fights with these brighter females, reducing interruption and allowing dominant females to successfully court more males as a result (Owens et al., 1994). Female Mormon crickets, *Anabrus simplex*, also compete against each other for access to males. In this species, the males provide females with protein-rich nuptial gifts. Females compete to gain access to these valuable reproductive resources, whereas males are selective, often rejecting female suitors (Robson & Gwynne, 2010). Status badge ornaments can be displayed in females across a range of taxa, indicating that similar selection pressures can drive female ornamentation across the animal kingdom.

Female ornaments can have a dual function in both attracting mates and deterring female competitors. This was shown to be the case in the pipefish species *Syngnathus typhle*. The female temporary ornament consists of a contrasting striped pattern that is used to attract males in courtship displays (Berglund & Rosenqvist, 2001). Berglund and Rosenqvist (2008) later found that this ornament was also used to intimidate rival females. Females reduced their own courtship displays and their willingness to engage in competition when faced with a highly ornamented female rival (Berglund & Rosenqvist, 2008). These examples indicate that female ornaments can evolve as dominance badges used in intrasexual competition. Ornamental traits that are favoured in competition may become subject to mate preference under the "armament-ornament" hypothesis, evolving a dual purpose; in both intrasexual competition and mate choice (Stern & Servedio, 2017), which form the basis of sexual selection.

Evolution through intersexual sexual selection

Sexual selection through male mate choice can drive the evolution of female ornaments (Amundsen et al., 1997; Cornwallis & Birkhead, 2007; Hopkins et al., 2015; Jones & Hunter, 1993; Murray et al., 2018; South & Arnqvist, 2011; Tigreros et al., 2014). Through male mate choice, males will select preferred females or alter their resources to each mate, leading to non-random mating and influencing the mating success of the female (Edward & Chapman, 2011). Male mate choice has been observed in a range of taxa including insects (see for example Bussiére et al., 2008; Hopkins et al., 2015; Murray et al., 2018; Robson & Gwynne, 2010; South & Arnqvist, 2011; Tigreros et al., 2014), birds (see for examples Amundsen et al., 1997; Cornwallis & Birkhead, 2007; Jones & Hunter, 1993) and fish (see for example Berglund & Rosenqvist, 2001), hence sexual selection can be found acting on females across the animal kingdom.

Ornaments may be derived from other evolutionary pathways before being subject to sexual selection. The sensory drive hypothesis, in which characteristics of the environment shape how signalling and sensory traits evolve with regards to their transmission and reception (Cummings et al., 2018; Ryan & Rand, 1990), is one such pathway that can both independently and, in combination with sexual selection, lead to the evolution of ornaments. For example, females from the lizard genus *Anolis* bear colourful dewlaps, an erectable throat flap. In species that occupied tree crowns or twigs, females had larger dewlaps, likely because the dense visual environment may require large dewlaps to catch the attention of a conspecifics or to overcome the high amount of background movement (Harrison & Poe, 2012). Sensory bias, which stems from sensory drive, is the evolution of sensory and signal traits and preferences that arise from non-sexual contexts (e.g., foraging) which go on to be favoured in sexual selection (Fuller et al., 2005). Sensory bias has been used to successfully explain the colouration in cichlids and female preference in túngara frog calls (Ryan & Rand, 1990; Seehausen et al., 2008). Another model example of sensory bias is the male sexual ornament in swordtail fish, which exploits the females pre-existing bias favouring large mating partners (Rosenthal & Evans, 1998). In the swordtail

characin, (*Corynopoma riisei*) males maintain a flag-like ornament which was found to covary with the female diet (Kolm et al., 2012). The shape of the ornament resembled an ant in populations where ants made up a large proportion of the female diet, demonstrating the male ornament closely matches the search images used during foraging (Kolm et al., 2012). Sensory drive can therefore both explain the evolution of some ornaments independently, or in addition with sexual selection via sensory bias.

Ornaments as signals

Ornaments are used as signals by the receiver to gain information needed to make the optimum mate choice decisions (Doutrelant et al., 2020). Female ornaments can signal a multitude of factors such as species identification, reproductive receptibility, female quality and fecundity (Hernández et al., 2021; Robart & Sinervo, 2018). These signals are beneficial to males increasing the chance of successful reproduction (Edward & Chapman, 2011; Kopena et al., 2020).

Female ornaments can be used for species recognition, a hypothesis proposed by Wallace (1889) where species-specific ornamental traits are used for species recognition beneficial for both sexes to avoid hybridization and aggression, which could be detrimental to the individual's fitness. An experiment using two species of Far Eastern dace (*Tribolodon hakonensis* and *Tribolodon sachalinensis*) found that males use female ornamentation, displayed during the breeding season, to visually recognize females of the same species (Atsumi et al., 2019). Similar results have been found in the cichlid fish species *Tropheus* in which both males and females courted partners from the same population significantly more than females from four allopatric populations (Zoppoth et al., 2013), suggesting the female colouration may have evolved through species recognition. Female ornaments can thus be related to the habitat reducing search time and preventing potential hybridisation.

Female ornaments can also convey information about reproductive receptivity, a useful signal to males, and beneficial for females to avoid male harassment during non-

reproductive times. Female mammals do not often display ornaments; however, some female primates have bright skin colourings which act as signals to the males regarding female receptibility (Rigaill et al., 2019; Rooker & Gavrilets, 2020). In Japanese macaques, the red skin colouring on the face and hindquarters of the females was linked to information about the reproductive cycle, social rank and potentially the body mass of the female prior to the breeding season (Rigaill et al., 2019). However, no evidence was found that males preferred brighter skin colouration or that males used the colouration to choose females based on their cycle (Rigaill & Garcia, 2021). Similarly, in the female ornamentation of convict cichlids, gold ventral colouration is displayed when females are reproductively receptive and is reduced after mating (Robart & Sinervo, 2018). These two examples demonstrate that female colouration may have evolved to signal information regarding reproductive receptibility to males. In some cases, females display ornaments during the non-reproductive time, for example in the damselfly species *Agriocnemis femina,* red coloration signals the sexual immaturity of the females, as females with these ornaments are lighter, carry immature eggs and mate less frequently (Khan, 2020). After a week, at which point they reach sexual maturity, females change into a less conspicuous green colour. The red colouration likely reduces sexual harassment during the non-reproductive stage of the female life cycle (Khan, 2020). Similarly, in the white-necked Jacobin hummingbird, 30% of the females display a male-like ornament, evolving for social harassment avoidance (Falk et al., 2021). Female ornaments can evolve as a signal of both reproductive receptibility and sexual immaturity, allowing the female to avoid costly male harassment and preventing the male from unprofitable mating during non-reproductive times.

Trade-offs and honesty of female ornaments

Females are often assumed to be constrained by ornaments, needing to reallocate resources away from reproduction to maintain ornamentation, lowering fecundity and quality (Fitzpatrick et al., 1995). For example, some female fence lizards*, Sceloporus undulatus*, bear male-like ornaments and are discriminated against during mate choice.

Ornamented females were found to have a lower reproductive output, as eggs were laid and hatched later than those of ornamented females, resulting in a cost of ornament expression (Swierk & Langkilde, 2013). Instead, the female ornament was found to correspond with female sprinting speeds, with offspring from ornamented females having longer body lengths and greater antipredator responsiveness. Ornamented females were, therefore, better at fleeing predatory attacks suggesting that ornaments may be selected in certain environments with high predation (Assis et al., 2018). In common yellowthroats (*Geothlypis trichas*) females with brighter, more saturated bibs showed a decrease in fecundity, laying fewer eggs and were more likely to lose the total brood due to predation (Freeman-Gallant et al., 2014). Ornaments can lead to a trade-off with fecundity, however in some cases can still signal beneficial traits to the receiver, causing ornaments to remain in the population.

Alternatively, many female ornaments can honestly signal the quality and condition of the individual. Factors such as stress, parasites, and disease have been found to negatively impact female ornamentation, therefore the appearance of the ornament can act as an honest signal of female health (Kopena et al., 2020; Lumpkin et al., 2014; Weiss, 2006; Weiss et al., 2013). Well-fed females have brighter ornaments in the ornate jumping spider species *Cosmophasis umbratical,* indicating that brightness was related to the health status of the bearer (Painting et al., 2017). Similarly, an increase in carotenoids, gained through diet, increases the saturation and hue of carotenoid-based colouration as represented in the Cyprinid fish species *Puntius titteya*, hence signalling the foraging abilities of the individual (Mieno & Karino, 2019). Likewise, a meta-analysis demonstrated that structural colouration in females can also represents their condition, such as body mass and immune function (White, 2020). Both males and females display a spotted ornament in spotted sandpipers, (*Actitis macularius*), a sex role reversed species. Female spot size was found to be affected by feather mite load, in which spot size decreased as mite load increased (Blizard & Pruett-Jones, 2017). Female ornaments are therefore honest signals of body condition such as body mass, parasite load, and beneficial traits such as foraging ability and predator avoidance, making them effective signals in mate choice.

In some species female ornaments can honestly signal fecundity, indicating that females do not always reallocate resources away from gamete production by displaying ornaments (Cornwallis & Birkhead, 2007; Cotton et al., 2015; Doutrelant et al., 2020; Hopkins et al., 2015; Simmons & Emlen, 2008; Weiss, 2006). For example, in female Iberian green lizards (*Lacerta schreiberi*), colour saturation was correlated with larger clutches, shorter incubation time and greater hatchling success, all of which are beneficial traits to males (Kopena et al., 2020). Similar results were found in the Alpine newt, (*Ichthyosaura alpestris*), with orange females being more fertile, producing larger clutches and having a faster laying speed, meaning that these ornamented females showed no trade-off with ornamentation and fecundity (Lüdtke & Foerster, 2019). Female cyprinid fish (*Puntius titteya*), who displayed ornaments with higher red saturation produced larger eggs (Mieno & Karino, 2019). Large eggs result in a higher survival rate of offspring, therefore providing direct benefits to males (Mieno & Karino, 2019). Body size and belly width was correlated with brood size in the black-finned goodeid (*Girardinichthys viviparus*); however, colour was a negative function of offspring survival (Méndez-Janovitz & Macías Garcia, 2017). Female blue tits that displayed bright yellow plumage transferred more carotenoids to eggs under immune challenging conditions, thus brighter females invested more in reproduction under these conditions compared to duller females (Midamegbe et al., 2013). Honest female ornaments have been found across a range of taxa, indicating that females do not need to reallocate resources away from reproduction to express ornaments, instead female ornaments signal traits beneficial to males. Males should thus favour these honest signals in mate choice as they provide benefits through increased offspring survival (Edward & Chapman, 2011; Kopena et al., 2020; Paczolt & Jones, 2010).

Conventional sex role species

Mate choice is a key selection pressure driving the evolution of female ornamentation in a diverse range of taxa, with both conventional and reversed sex roles. In conventional sex role species, in which sexual selection acts more strongly on the male sex, male mate choice is influenced by factors such as variation in female quality, the number of

available females to a single male, cost of mating effort (i.e., has the male invested a lot in his own sexually selected traits) and the capacity of the male to mate with all receptive females (Edward & Chapman, 2011). If the female ornament is an honest signal of fecundity, the male will directly benefit through increased offspring development and survival (Tigreros et al., 2014). Displaying ornaments can also benefit females, as females can acquire more sperm from high quality, dominant males, which is likely to provide reproductive benefits (Cornwallis & Birkhead, 2007).

A meta-analysis on female bird ornamentation found an association between colourful female traits, female quality (immune response and body mass), fecundity (clutch size), and male mate preference, suggesting that female traits are likely under sexual selection (Hernández et al., 2021). Indeed, studies have found males to prefer more ornamented females in a number of bird studies, and ornaments were correlated with female quality, such as colour in blue throats (*Luscinia s. svecica)* (Amundsen et al., 1997), large combs in fowl species (*Gallus gallus)* (Cornwallis & Birkhead, 2007), and crest size in crested auklets (*Aethia cristatella)* (Jones & Hunter, 1993). Similar mate choice patterns have been observed in a range of taxa, indicating a general trend in male mate choice for ornamented females. For example, male cyprinid fish, *Puntius titteya,* were found to prefer females with brighter red colouration like the preference exhibited by females (Mieno & Karino, 2019). Likewise in several insect species, males were also found to prefer more ornamented females; including in butterflies (Tigreros et al., 2014), glow worms (Hopkins et al., 2015), mosquitos (South & Arnqvist, 2011) and crickets (Robson & Gwynne, 2010)). Female ornaments can therefore evolve independently under sexual selection, through male mate choice, in a diverse range of mating systems.

Sex role reversed species

Females from sex role reversed mating systems are often more ornamented than males, expressing exaggerated and elaborate traits. Under sex role reversal, females compete for mating opportunities and sexual selection acts most strongly on the female sex,

which can cause the evolution of ornamental traits (Fritzsche et al., 2021; Hare & Simmons, 2019). Differences in mating competition can also coevolve with other traits such as parental care, which can contribute to the strength and direction of sexual selection (Edward & Chapman, 2011; Fritzsche et al., 2021). In sex role reversal males are considered the choosers, discriminating against certain females and preferring others. For example, in the sex role reversed dance fly species *Rhamphomyia longicauda*, males exhibit a strong preference for certain female ornaments (Bussiére et al., 2008; Murray et al., 2018). One study found that males preferred females with larger pinnate leg scales and inflatable abdominal sacs, hence these female ornaments are used during mate choice (Murray et al., 2018). In another study, males showed preference for large wings and short tibiae in the same species (Bussiére et al., 2008). Ornaments have evolved in some dance fly species, such as *Empis aestiva,* as these females heavily rely on the nuptial gifts for egg development (Hunter & Bussière, 2019). Likewise, in the sex role reversed butterfly species *Bicyclus anynana,* males were reluctant to mate with unornamented females, showing a preference for ornamentation (Ng et al., 2017). Preferences for female ornamentation are also prevalent in Syngnathids, a fish family containing many sex-role reversed species. For example, in the sex role reversed pipefish species, *Syngnathus typhle,* females are the more ornamented sex. Males were found to prefer more ornamented over unornamented females (Berglund & Rosenqvist, 2001). These examples demonstrate that intersexual sexual selection can contribute to the evolution of female ornaments in sex role reversed species.

Sex roles can be dynamic - in some species the strength of sexual selection acting on males and females fluctuates throughout the breeding season (Hare & Simmons, 2021). Changes in the OSR (the proportion of reproductive males to females) from male-biased to female-biased can alter the strength of sexual selection on either sex (Clutton-Brock & Vincent, 1991). A model system of this is the two-spotted goby, *Gobiusculus flavescens*, which demonstrate a complete reversal of sex roles during the breeding season (review in Amundsen, 2018). At the start of the breeding season, the OSR is heavily male-biased, resulting in male-male competition and choosy females. During this time sexual selection is acting on the male sex and roles are conventional. Towards the end of the breeding season, females are in excess leading to a female biased OSR. Females become more competitive, engage in courtship, and show less preference in mate choice. As a result, both sexes

maintain ornamental colouration. A similar scenario has been observed in the bush cricket, *Kawanaphila nartee*, where sex roles are reversed early in the season when resources are scarce and change to conventional roles later when food is abundant (Hare & Simmons, 2021). Therefore, sexual selection drives sexual dimorphism in ear size, in which female bush crickets have larger, more open ears (auditory spiracles) compared to males (Hare & Simmons, 2021). These species display the variability of sexual selection and how quickly sexual behaviours can change due to short-term fluctuations in mating competition.

Sex role reversed species have provided a breakthrough for sexual selection research, highlighting that sexual selection can act not only on males but also females, whilst demonstrating that mate competition is a continuum (Fritzsche et al., 2021). Future research should focus on examining sexual selection in both males and females across a phylogenetically diverse range of species. By investigating the impacts of ecological, demographic and life-history factors on sexual selection, we can gain a greater appreciation for the variability and plasticity of sex roles.

Syngnathidae family as a study species

Syngnathidae (pipefish, seahorses and seadragons) are a unique family, as all species have evolved male pregnancy. Pipefish are found worldwide and contain a high number of sex role reversed species which display female ornamentation, making them an ideal model species for sexual selections studies. Male pipefish incubate eggs in special brood pouches attached to the ventral surface of the body, providing embryos with aeration and nutrients. This causes males to invest a lot of time and energy into offspring (review in Stölting & Wilson, 2007). In some species males become choosy in mate choice, favouring high-quality fecund females. As a result, sexual selection acts on the female sex, resulting in the wide variety of female ornaments present in several pipefish species (e.g., *Syngnathus typhle, Syngnathus scovelli*, *Nerophis ophidion* and *Stigmatopora nigra*) (Anderson & Jones, 2019; Berglund, 2000; Berglund et al., 1986; Mobley et al., 2018; Rosenqvist & Berglund, 2011). These female ornaments were found to be under male mate preference (Anderson & Jones,

2019; Berglund & Rosenqvist, 1993; Berglund & Rosenqvist, 2001; Partridge et al., 2013), female-female competition (Berglund & Rosenqvist, 2008; Monteiro et al., 2002; Rosenqvist & Berglund, 2011), and were honest signals of fecundity (Berglund et al., 1997; Cunha et al., 2017; Mobley et al., 2018). Pipefish can therefore be used to explore sexual selection and the underlying assumption of female ornamentation (i.e., trade-offs with fecundity). The continuum of mating systems within pipefish allows us to compare clades to gain a better understanding of the evolution of mating systems and the plasticity of sex roles (Fritzsche et al., 2021). Incorporating ecological, geographic, and life-history factors into these studies will add a richer understanding to mating-systems and the role and evolution of female ornaments.

Stigmatopora nigra and rationale for my study

Stigmatopora nigra is a species of pipefish found throughout Australia and New Zealand (Kuiter, 2000). Females have exaggerated ornaments consisting of a dorsoventrally flattened belly with light and dark stripes running parallel to each other (Kuiter, 2000; Mobley et al., 2018), see Figure 1. This exaggerated ornament differs from most pipefish species which have deeply keeled bodies, skin folds and different markings and colouration (Berglund, 2000; Berglund et al., 1986; Rosenqvist & Berglund, 2011). New Zealand *S. nigra* populations have been found to have either orange or yellow stripes, differing between populations (S. Flanagan, personal observation). Little is known about the ornamentation of *S. nigra* and whether it is used primarily to attract males during courtship and/or for intrasexual competition (Mobley et al., 2018). Courtship displays have not yet been described; thus, the life-history of this species is largely unknown. My study will aim to fill some of these knowledge gaps by describing the courtship display, the role each sex plays in courtship and identifying whether the female ornament undergoes sexual selection. Overall, this information is relevant to the field as it can be compared to other pipefish studies and will shed light on the evolution of female ornaments, adding new knowledge to our understanding of ornaments, pipefish, and this field of research in general.

The aims of my research are to:

Assess the courtship displays of *S. nigra* in terms of the role of each sex and how individuals interact in a 'natural' setting created through mesocosm experiments (Chapter 2)

Identify whether there is geographic variation in the female ornament and whether female body size and/or ornament are honest signals of female fecundity (Chapter 3).

My data chapters will be written in a scientific paper format as I intend to publish papers from this work.
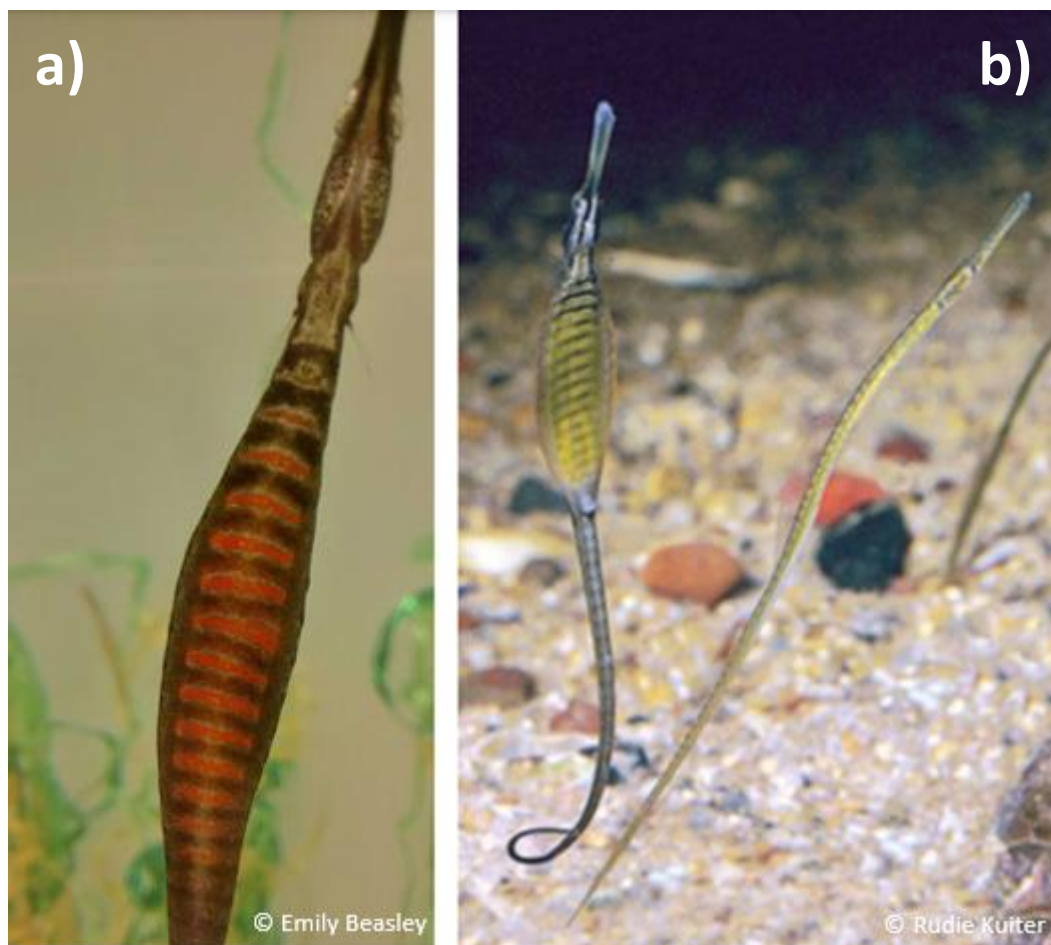
Figure 1. a) New Zealand S. nigra (female) and b) Australian S. nigra, female (left) and male (right) Photography © Emily Beasley (a) and Rudie Kuiter (b)

Chapter 2: Exploring the role of female ornament and courtship behaviours of the wide-bodied pipefish, *Stigmatopora nigra*

Abstract

Female ornaments are present in a range of taxa and, like male ornaments, can evolve through sexual selection. Observing phenotypic variation alone is not sufficient to determine whether the trait is used in courtship, highlighting the importance of studying courtship behaviours in sexual selection studies. By observing courtship behaviour, we can determine whether female ornaments play a role in male mate choice, female-female competition, and the role of each sex during courtship. The wide-bodied pipefish, *Stigmatopora nigra*, display an exaggerated female ornament, however little was known about the ornament and whether it is used in courtship and or competition. I predicted that this species would be sex role reversed, hence sexual selection would act most strongly on the female sex, resulting in the female being most active in courtship. Ten mesocosm experiments, each containing 8 females and 8 males, were used to explore the role of this female ornament during courtship. I found that female ornamental displays were mostly directed towards males, but occasionally were also directed towards other females. To my surprise, and against sex role reversal expectations, males were the most frequent initiator of courtship (89%), displayed aggressive chasing behaviour, and were often observed courting in a group of multiple males to one female. Overall, the results from this study suggest that the female ornament of *S. nigra* is important in male mate choice and has likely evolved through sexual selection. My surprising results paint a nuanced picture of sex role reversal in this species, raising intriguing questions about the sensitivity of courtship behaviours to social cues such as density and sex ratio.

Introduction

Elaborate ornamentation exists across the animal kingdom, bearing some cost to the carrier and serving no purpose to survival (Kraaijeveld, 2019; Kraaijeveld et al., 2007). Ornaments encompass the diverse range of colouration and exaggerated body morphology, such as the iridescent blue spots expressed by male two-spotted goby (*Gobiusculus flavescens*) (Amundsen, 2018), evolving through sexual selection and competition pressures. Male ornaments act as signals, honestly indicating the reproductive quality, good genes and/or competitive abilities of the individual (de Zwaan et al., 2019; Ferrer et al., 2015; Ogita & Karino, 2019). For example, male common yellowthroats (*Geothlypis trichas*) with larger black facial masks had improved parasite resistance and survival (Dunn et al., 2013). Similarly, male black grouse (*Lyrurus tetrix*) with larger eye comb ornaments were more dominant and in better condition (Harris et al., 2018). Potential mates or rivals can therefore use honest ornamental traits as signals, altering their behaviour and decisions based on the quality of the ornament (Dougherty, 2021). Some ornaments can also have a dual function both in attracting mates and in contest with rivals (Rometsch et al., 2021).

Female ornaments are expressed in a range of taxa, and in some species are more elaborate than the male sex. Female sexual traits, like male ornaments, can evolve through sexual selection (i.e., mate choice and/or intrasexual competition), which influences the females' mating success (reviewed in Hare & Simmons, 2019). Alternatively, female ornaments can evolve through resource competition (e.g., food), correlated inheritance with male ornaments, or as signals of sexual receptivity (Clark & Rankin, 2020; Rigaill et al., 2019; Ziegelbecker et al., 2018). It is becoming increasingly evident that female ornaments are beneficial to the bearer, evolving through multiple pathways (Doutrelant et al., 2020). However, one assumption that has delayed female ornament research in the past, is the premise that females invest more in gametes, hence expressing ornaments would lead to a trade-off with fecundity (Fitzpatrick et al., 1995). A trade-off due to ornamentation has been noted in several female sexual selection studies, with females expressing ornaments having a lowered fecundity (Assis et al., 2018; Freeman-Gallant et al., 2014; Swierk & Langkilde, 2013). Other studies have found the opposite effect, where female ornaments act as honest signals of fecundity, influencing male mate choice, and therefore evolving through sexual selection (Kopena et al., 2020; Lüdtke & Foerster, 2019; Mieno & Karino, 2019). Using

females to study sexual selection concepts is therefore beneficial to 1) determine whether female ornaments are favoured in mate choice and 2) to address the extent to which fecundity requires trade-offs with display traits and whether females can display honest signals.

Sex role reversed species provide an opportunity to test whether females face greater trade-offs than males when expressing ornamentation (Fritzche et al., 2021; Hare & Simmons, 2019). Under sex role reversal we would expect females to compete for mates and males to be choosy. By studying male mate choice, we can identify whether males show preference in female ornaments and if ornaments correlate to female quality (e.g., body condition and parasite load) or fecundity. Using sex role reversed species we can better understand mating systems, how they have evolved, and whether female ornaments are honest signals.

Syngnathidae (seahorses, seadragons and pipefish) are a unique family, all of which have evolved male pregnancy, making them an excellent study species for sexual selection (Anderson & Jones, 2019; Berglund & Rosenqvist, 2001; Bernet et al., 1998; Flanagan et al., 2014; Partridge et al., 2013). Males incubate the eggs in either a brood pouch or on the ventral body surface (Berglund et al., 1986; Goncalves et al., 2015). Males provide developing embryos with aeration, osmoregulation, and nutrients (review in Stölting & Wilson, 2007). Once embryos have fully developed, live young are released from the pouch. The lengthy pregnancy of males often exceeds the time the females require to produce a new clutch of eggs, hence the females become reproductively limited by the males, resulting in a female biased operational sex ratio (OSR) (Berglund & Rosenqvist, 1990; Berglund et al., 1986). The low number of reproductively receptive males limits female reproductive success, resulting in female-female competition for access to mates and male mate preference (Clutton-Brock, 2009; Clutton-Brock & Parker, 1992). Some species have evolved female ornaments, providing an excellent opportunity to study factors influencing female ornament evolution by sexual selection.

Courtship behaviours have been described for a several pipefish species, including *Syngnathus typhle, Nerophis lumbriciformis*, *Syngnathus abaster*, *Syngnathus scovelli*,

Syngnathus acusimilis (now Syngnathus schlegeli) and *Urocampus nanus*, providing valuable information about the role of each sex during courtship (Berglund & Rosenqvist, 1993; Kornienko, 2001; Monteiro et al., 2002; Partridge et al., 2013; Silva et al., 2006; Sogabe et al., 2012). Many pipefish display an array of female ornaments including deeply keeled bodies, skin folds, bands, stripes, and colouration (Berglund, 2000; Berglund et al., 1986; Rosenqvist & Berglund, 2011). Female ornamentation can either be temporarily amplified during courtship such as in the species *Syngnathus typhle*, or a permanent ornament visible throughout the year such as the species *Urocampus nanus* (Bernet et al., 1998; Sogabe & Yanagisawa, 2007). Sexual dimorphism in body size has also been expressed in many pipefish species, where females are larger than males (Winkler et al., 2012). These morphological differences between sexes make pipefish a great study species to explore the extent that sexual selection drives sexual dimorphism, and whether females bear any fitness cost in ornament expression.

Male reproductive rate is directly influenced by female quality in some pipefish species, therefore driving males to be selective in their mate choice. In female pipefish, body size and ornamentation influences selection preferences, with males expressing strong preferences for large, ornamented, and active females (Anderson & Jones, 2019; Berglund & Rosenqvist, 1990, 1993; Berglund & Rosenqvist, 2001; Berglund et al., 1986; Bernet et al., 1998; Flanagan et al., 2014; Partridge et al., 2013). Both body size and ornamentation have been found to correlate to fecundity and dominance, therefore signalling female quality (Berglund & Rosenqvist, 2001; Berglund et al., 1997; Flanagan et al., 2017; Mobley et al., 2018; Paczolt & Jones, 2010). By mating with preferred partners, males will gain direct benefits, as offspring will be of higher quality (Sandvik et al., 2000).

In sex role reversed species, where males are selective in mate choice, females can still remain discriminatory towards males, demonstrating mutual mate choice (Hare & Simmons, 2019). Mutual mate choice, when both males and females are selective in their mate partners, occurs in a range of different taxonomic groups (Dosen & Montgomerie, 2004; Jones & Hunter, 1993; Schlupp, 2018). Some studies have revealed mutual mate choice existing in several pipefish species, where females prefer to mate with larger males, as these males invest more energy per offspring, have larger pouches and can carry and care for heavier embryos (Berglund et al., 2005; Flanagan et al., 2017; Goncalves et al., 2015;

Nygård et al., 2019). Despite the occurrence of mutual mate choice in pipefish, mate preference displayed by females is not as strong as preference exhibited by males (Berglund & Rosenqvist, 1993), however strength of preference might fluctuate depending on the social environment.

Female ornamentation can be advantageous in female-female competition during pipefish courtship displays (Berglund & Rosenqvist, 2008). During courtship other females often try to disrupt the courting pair, thus females must compete with one another to defend their mate, hence displaying their ornaments more in the presence of other females as seen in *N. lumbriciformis* and *S. typhle* (Berglund & Rosenqvist, 2001; Berglund et al., 1997; Bernet et al., 1998; Monteiro et al., 2002). Highly ornamented females were found to dance more and as a result were more competitive, achieving greater mating success and transferring more eggs than non-ornamented females (Berglund & Rosenqvist, 2001; Berglund et al., 1997; Bernet et al., 1998). The female ornament may therefore have a dual function in both male mate choice and female-female competition.

Stigmatopora nigra is a species of pipefish found widely throughout Australia and New Zealand (Kuiter, 2000), yet its behaviour is unstudied. Females have exaggerated ornaments consisting of a dorsoventrally flattened belly with light and dark stripes running parallel to each other, differing from most other pipefish (Kuiter, 2000; Mobley et al., 2018). Little is known about the role of this female ornament, whether it is used primarily to attract males during courtship and/or for intrasexual competition (Mobley et al., 2018).

Many pipefish studies have used dichotomous choice tests to assess courtship behaviours and mate preference (Anderson & Jones, 2019; Berglund, 1995; Berglund & Rosenqvist, 2001; Berglund et al., 1997; Bernet et al., 1998; Partridge et al., 2013; Rosenqvist & Johansson, 1995; Sandvik et al., 2000). Dichotomous choice tests prevent individuals from interacting together. The number of individuals used in such a test is often small and does not represent a natural situation. In the wild some pipefish females display in lek-like groups, with males responding to attractive females. Larger, dominant females will often disrupt the mating process of smaller females (Berglund, 2000; Rosenqvist & Berglund, 2011). These behaviours may not be observed in choice tests due to the small group size and barriers. To overcome this, I will be using a mesocosm approach to analyse the courtship behaviour of *S. nigra*, reflecting a more natural environment with multiple

individuals interacting together. I will identify 1) what behaviours are associated with *S. nigra* courtship displays, 2) which sex initiates courtship and 3) differences in activity between sexes during courtship. This will greatly expand on the life-history knowledge of *S. nigra*, offer comparative analysis with other pipefish species, and give indications on how this novel female ornament may have evolved.

Methods

Pipefish collection and care

Male and female wide-bodied pipefish, *Stigmatopora nigra*, were collected in November 2020 at two sites in Tauranga harbour. 38 female and 60 males were collected from the Matua site (Latitude -37.66255, Longitude 176.10827) and 62 females and 60 males were collected from the Plummers point site (Latitude -37.66173, Longitude 176.04525). Fish were caught using a handheld seine net, stored overnight at the University of Waikato Marine Ecology lab, and transported to the University of Canterbury by car. Once back in Christchurch pipefish were acclimatised to the tanks in the aquarium at the University of Canterbury. For full pipefish transportation and care methods see supplementary material.

15 tanks were set up on two flow-through systems to house the pipefish. The 10 tanks used in the mesocosm experiment were made from modified transparent plastic storage containers. White plastic was taped to either side of the tank, to act as a visual barrier (like in Anderson & Jones, 2019). The other 5 tanks were used for housing excess pipefish or as holding tanks when sexes needed to be kept separate. The temperature for both systems was set to 18°C, comparable with the water temperature in Tauranga during the summer. Tank water was filtered through biofilters, and system was regularly topped up with fresh seawater. Fake seagrass plants were placed in tanks as refuge for the pipefish. Lights were set to a 12hr daylight cycle. Pipefish were fed live *Artemia spp*. twice daily. Tanks were cleaned daily removing any organic matter.

Males and female from each site were housed separately before entering the experiment. Each housing tank contained 20 or fewer pipefish. Males were held in housing tanks until live young had hatched from their brood pouch before entering the experiment (similar to Kornienko, 2001; Rose et al., 2013). Before entering the mesocosm experiment, pipefish were measured (total body length) and photographed.

Study design

In total 10 mesocosms were used during the experiment. Each mesocosm contained 8 female and 8 male pipefish. The 16 pipefish total was a combination of individuals from both sites. Mesocosms simulate a natural environment with multiple individuals interacting, allowing both courtship behaviours, and female-female competition to be observed. Several studies have used mesocosms in a range of behavioural fish studies (Desjardins et al., 2001; Silva et al., 2006) to analyse how individuals interact together.

The start dates for the mesocosms were staggered with 2 trials starting on the same day every one to two days. Males and females were combined in same tank at 8am on the start date.

Filming behaviours

Panasonic HC-V180 Camcorders, mounted on tripods were used to film the mesocosm tanks. Video recording behaviours is common in pipefish courtship studies (Anderson & Jones, 2019; Berglund & Rosenqvist, 1990; Berglund & Rosenqvist, 2001; Berglund et al., 1997; Berglund et al., 2005; Partridge et al., 2013). Each tank was filmed from 8am to 10am, which captured the dawn period as aquarium lights gradually ramped-up from 7:45 am onwards, a second filming session was held from 12 pm to 1pm. Other Syngnathid studies have noted an increase in courtship just after dawn (Masonjones & Lewis, 1996; Matsumoto & Yanagisawa, 2001; Monteiro et al., 2002; Partridge et al., 2013), therefore I filmed for 2 hours during this time to ensure as much courtship behaviour was captured as possible. I also carried out a filming session from 8 to 9pm (when aquarium lights go off) for the first 3 nights of the experiment. No courtship or other active behaviours

were observed over these three nights, similar to findings to Monteiro et al. (2002). As a result, I removed this filming session entirely. In total each mesocosm was filmed for 3 hours daily for 7 consecutive days, resulting in 210 hours of video footage.

Activity budget of general behaviours

A scan-sampling technique was used to create a time budget for pipefish. After observing pipefish in the tanks and reviewing subsets of the recorded videos, an ethogram was created with all observed behaviours displayed by pipefish (Table 1). Videos were scored at 15-minute intervals (like Berglund et al., 1997). At each 15-minute time point, pipefish were watched for 10-30 seconds and the number of fish showing each behaviour was recorded. If courtship was observed, the number of females and males in each courting group was also recorded. When courtship was partially out of sight, the sex of the individuals involved were difficult to determine and therefore recorded as unknown. A note was also made when female-female interactions were observed. In total there were 8 samples from each AM video and 4 samples from each Noon video, for a total of 835 scan samples across all days and trials. This was 5 samples less than expected, due to camera failing issues during one of the recording sessions.

I used a Principal Components Analysis to determine interscorer reliability for scan sampling scoring. 24 videos, including both AM and noon samples across trials and days, were rescored and compared with their corresponding data from the original scan sampling. The ordination was visually assessed to observe any patterns. I performed a Procrustes analysis to compare the original and rescored data and used the protest function to measure the significance of the Procrustes statistic (vegan package version 2.5-7) (Oksanen et al. 2020).

Visible and partially visible courtship were combined as one behaviour, courtship, as mean proportion of individuals displaying each behaviour did not differ greatly from each other (Wilcoxon rank sum test= 303, p-value = 0.1472). I removed all zero proportions from the data set, as these represented behaviours that were not observed in the sample. Since I was only interested in what behaviours pipefish displayed, I chose to remove the behaviours not observed in the sample. All data analysis was performed in R version 4.0.5 (R Core Team

2021). To determine whether pipefish displayed certain behaviours more than others, the mean proportion of individuals displaying each behaviour was compared using a Kruskal-Wallis rank sum test. A Dunn's test (post hoc test) was then used to determine which behaviours differed significantly from each other.

Table 1. S. nigra behaviours recorded during scan-sampling video data

Behaviour	Description
Courtship visible	Active courtship behaviours (wiggles and poses) observed in clear view
Courtship partially visible	Active courtship behaviours observed with view obstructed
Chasing	Fish swimming parallel to one another at speed
Directional Swimming	Pipefish swimming horizontally, at least double its body length in distance
Active movement	Moving and exerting a lot of energy without any direction
Non-active movement	Drifting, stationary, minor movements
On plant	Holding on to fake sea grass (or sometimes airline hose) with tail
Cleaning	Curling tail around body and moving tail down body with speed, curl and uncurl
Copulation	Transfer of eggs from female to male
Holding tails	Pipefish using tail to hold another pipefish's tail
Interaction	Action of one pipefish causing reaction in another e.g., touch
Floating	Pipefish floating on the surface of the water
Out of sight	Pipefish could not be seen

Courtship behaviour analysis

Courtship was analysed to understand i) which behaviours are associated with *S. nigra* courtship displays, ii) the role of each sex during courtship with respect to initiation and display duration and iii) whether female ornamental displays are used for courtship

and/or female-female competition. Videos with courtship present were extracted from the scan sampling analysis as a starting point for further analysis. BORIS (Behavioural Observation Research Interactive Software) (Friard & Gamba, 2016) was used to calculate exact durations of observed behaviours, a technique used in other pipefish studies (Berglund & Rosenqvist, 2001; Berglund et al., 2005; Partridge et al., 2013). An ethogram was created in BORIS, with relevant behaviours observed in courtship (Table 2). Point events were one off instantaneous behaviours (no duration), whereas start and stop time stamps were recorded for state events, allowing duration to be calculated. Both wiggles and poses had 3 associated modifiers which were recorded every time a pose or wiggle was observed (Table 3). Courtship behaviours were split into male and female behaviours and each group scored separately. Due to the nature of the study design, individuals were unable to be identified in the video, hence behaviours were scored by sex rather than based on the individual. Female-female interactions were also observed, in which case females where split into 'female' and 'second female'. If multiple courting events occurred simultaneously within the tank, that segment of video was re-scored to record the second courting group. To remain consistent, courtship events were considered finished when pipefish in the courting group had displayed non-active behaviours for more than 60 seconds or if courting individuals had moved more than two body lengths away from each other.

Table 2 Ethogram of active and non-active courtship behaviours

Behaviour code	Behaviour type	Description
Wiggle	State event	Pipefish moves upper body from side to side.
Follow	Point event	Male follows female slowly
Pose	State event	Female poses to recipient
Move away	Point event	Pipefish moves away from counterpart
Touch	Point event	Pipefish intentionally touches other pipefish with snout
Inactive	State event	Pipefish is not moving
Holding tails	State event	Pipefish is using tail to hold onto another pipefish
Swim away	State event	Pipefish actively swims in a direction to get away from other pipefish in the group
Chasing	State event	Pipefish are swimming parallel to each other at speed
Leave group	Point event	Pipefish leave the group once scoring has begun

Surrounding	State event	Pipefish are surrounding and closing in on counterpart
Out of sight	State event	Pipefish not visible

Table 3 Active courtship behaviours with corresponding modifiers used in BORIS

Behaviour	**Modifier 1**	**Modifier 2**	**Modifier 3**
Pose	Recipient: Male | Female	Type of pose: S-pose| non-s- pose| Flash	Side displayed: Front | back
Wiggle	Recipient: Male | Female	Type of wiggle: Active | non-active	Number in courting group

The excel sheets exported from BORIS were read into R (v 4.0.5), combined and durations of each behaviour calculated. The total duration of each courtship event was also calculated and given a unique label. For each courtship event, the sex that initiated courtship was identified by recording whether the first active courtship behaviour (pose or wiggle) was performed by a male or a female (like Partridge et al., 2013). The proportion of reciprocated courtship (i.e., when both sexes displayed active courtship behaviours in the courting bout) events initiated by males (rather than females) was compared to a null expectation of 0.5 using a one-proportion z-test. To determine whether females displayed the ornament more frequently during intersexual or intrasexual interactions, a one-proportion z-test was used to compare the proportion of female displays performed towards males (as opposed to towards females) to a null expectation of 0.5. I analysed unreciprocated courtship events, when one sex display with no response from the other, using a chi-squared test, to determine if this behaviour was more frequent for one sex compared to the other. To determine whether the pose and wiggle behaviours were performed more frequently than expected in one sex or the other, a chi-squared test was performed.

A linear mixed model (lmerTest package, R version 4.0.5) (Kuznetsova et al., 2017) was used determine whether group size impacted male wiggle duration. Prior to running the model, courtship events involving group sizes with 5 or more males were combined into one category, as there were a limited number of observations within these large groups. Two extreme durations were removed to avoid undue influence of outliers. Duration was log transformed to meet normality assumptions. Log duration was the response variable, group size was a fixed effect and courtship event was a random effect. A post hoc test was

performed to do pairwise comparisons of the levels within the group size factor (emmeans package v 1.7.2) (Russell 2022).

A mixed effect model was also used to predict the proportion of time spent actively courting within a courtship bout based on sex, time of day (AM and Noon) and day filmed (1- 7 days) using the lmerTest package (Kuznetsova et al. 2017) in R (version 4.0.5). Courtship event nested within trial was included as a random effect. Model assumptions were analysed using quantile-quantile plots to check for normality. Only courtship events where both females and males were observed actively courting were included. For significant relationships between the fixed effects and the response variable, a post hoc test (emmeans package v 1.7.2) (Russell 2022) was used for pairwise comparisons between each level of the factors.

Results

Description of S. nigra courtship behaviours

The overall pattern observed through scan sampling was that pipefish mostly displayed non-active behaviour, but when pipefish were seen displaying another behaviour it was mostly associated with intersexual interactions (i.e., courtship and chasing behaviour) (Figure 3). All behaviours in Table 1 were observed during scan sampling except for copulation. Despite capturing many courtship events and having 26 pregnancies in the mesocosm trials, copulation was not recorded at all. All observed behaviours are described in detail below.

Visual inspection of the ordination plots indicated that original and resampled data from scan sampling were closely clustered together, suggesting that there was little change in scoring technique. The Procrustes analyses showed that there is a significant correlation (m2 = 0.64, correlation = 0.60, p = 0.001) between the original and rescored data, confirming that scoring was reliable.
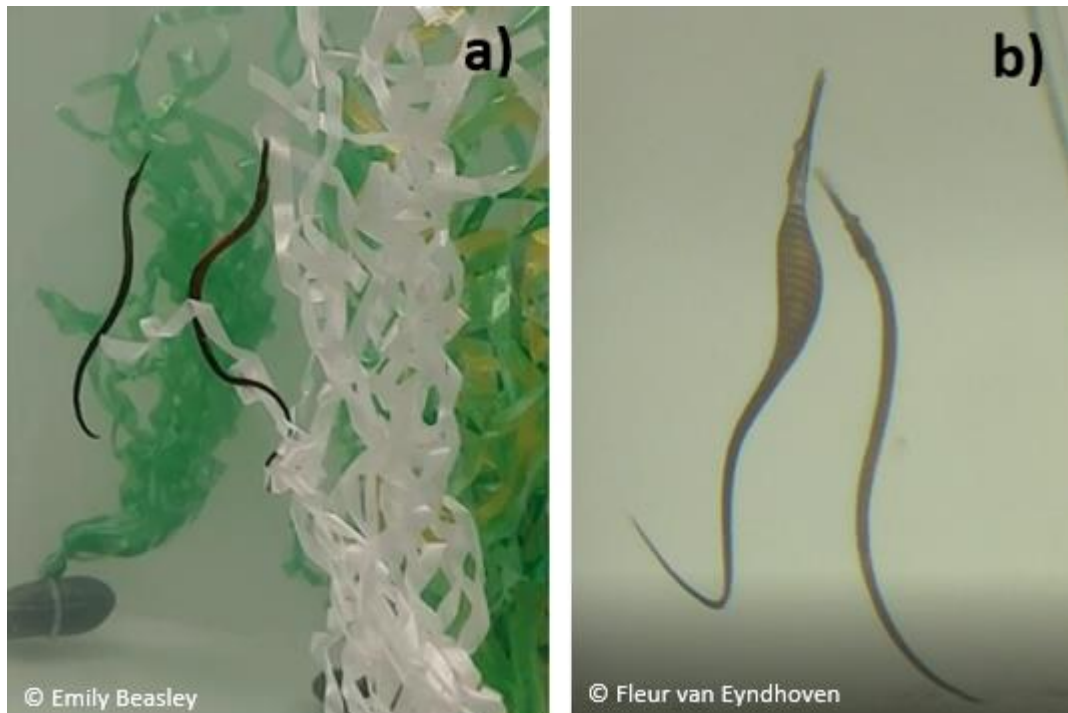
Figure 2 S. nigra courtship behaviours. a) left: male observing female and right: female s-posing, b) left female posing displaying ornament and right male wiggling in response. Photography © Emily Beasley (a) and Fleur van Eyndhoven (b)

S. nigra had two distinctive courtship behaviours, wiggles and poses. Wiggles were side to side movement of the upper half of the body, displayed facing the recipient in a vertical position (Figure 2). Both males and females were seen displaying wiggle behaviour, but males were observed wiggling more frequently (Table 4). Male wiggles were directed at females, with no observations of males wiggling towards other males. Wiggles were categorised as active, when movement was vigorous with large side to side movement, or subtle, when movement from side to side was slower and less extreme. During active wiggles males tended to encroach in on the female of interest. Males in the courting group were not often seen wiggling at the same time, instead after one male had stopped wiggling, another male would start. Females were seen wiggling towards both males and females (Figure 4). Female wiggling consisted of side-to-side movement of the belly, carried out with some speed to create a shimmy.

Table 4 Number of poses and wiggle behaviours displayed by females and males.

Behaviour	Female	Male	χ^2	P-value
Wiggle	189	1760		
Pose	873	1	2092.3	<2.2e-16

The more prominent female courtship behaviour was the pose, which consisted of three variations: the s-pose, non-s pose and a flash. The s-pose consisted of the female arching her back causing the head to drop back and the belly round forward (Figure 2). This ensured that the ornament was in full display towards the recipient. Posing behaviour was seen in both courtship and female-female interactions. The two other pose types were flashes and non-s-poses in which either the dorsal or ventral side was displayed. Flashes were fast flickers of the ornament directed towards the recipient. Non-s-poses were when females remained vertical and appeared to spread out their torso, exaggerating the wideness of their belly. Non-s-poses were used to display both, ventral side, where ornament was shown, and dorsal side, where dark dorsal surface accentuated the width of the belly. Both flashes and non-s-poses were not seen as frequently as s-poses (S-pose: 694, non-S-pose:202 and flash: 13).

Chasing was seen on a few occasions and could can be related to the parallel swimming observed in other pipefish courtship displays (Kornienko, 2001; Silva et al., 2006; Sogabe et al., 2012). Males were observed swimming parallel to female with speed. It appears chasing occurred when females were attempting to escape a large group of males.

Surrounding was another form of courtship behaviour recorded for this species. During surrounding behaviour, multiple males would surround the female encroaching on her until she would move away or display. Surrounding also encompassed males following females around without the parallel formation and speed categorised by chasing behaviour.

Activity budget

Pipefish displayed some behaviours more frequently than others (Kruskal-Wallis chi-squared = 1984.6, df = 11, p-value < 0.0001). Several comparisons were found to be significant (Appendix B, Table 9) but most notably, non-active movement was significantly different compared to all other behaviours (Figure 3).
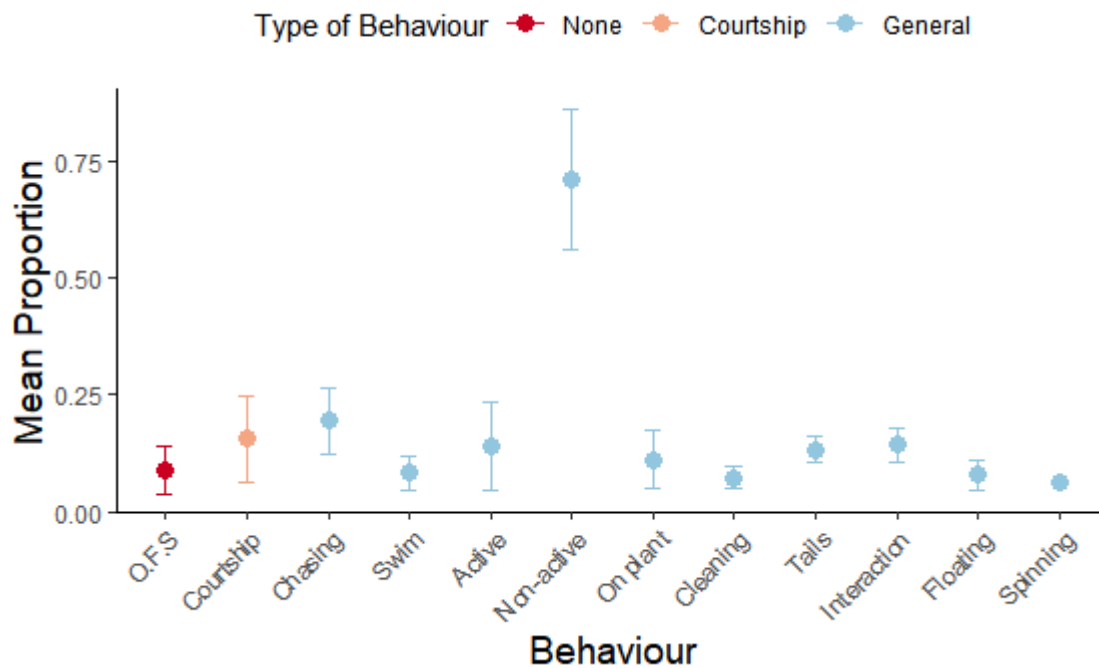
Figure 3 Mean proportion of individuals displaying each behaviour calculated from all samples. Behaviours categorised into type: courtship displays (orange), general or non-courtship behaviours (light blue) and out of sight (red)

Courtship behaviours

Males initiated reciprocated courtship significantly more frequently than females (p-value <0.001, proportion= 0.89). From the 85 reciprocal courtship events scored, males initiated 89% of these. In displays where courtship was unreciprocated both sexes were equally common to initiate failed courtship events (X^2 = 0.34, df = 1, p-value = 0.56).

Females displayed their ornament significantly more towards males than towards other females (p-value= < 0.001, proportion= 0.94). 94% of female displays were directed towards males of which 82% of the displays were poses and 18% being wiggles (Figure 4). Only 6% of displays were directed at females, of which 46% were wiggles and 54% were poses (Figure 4).
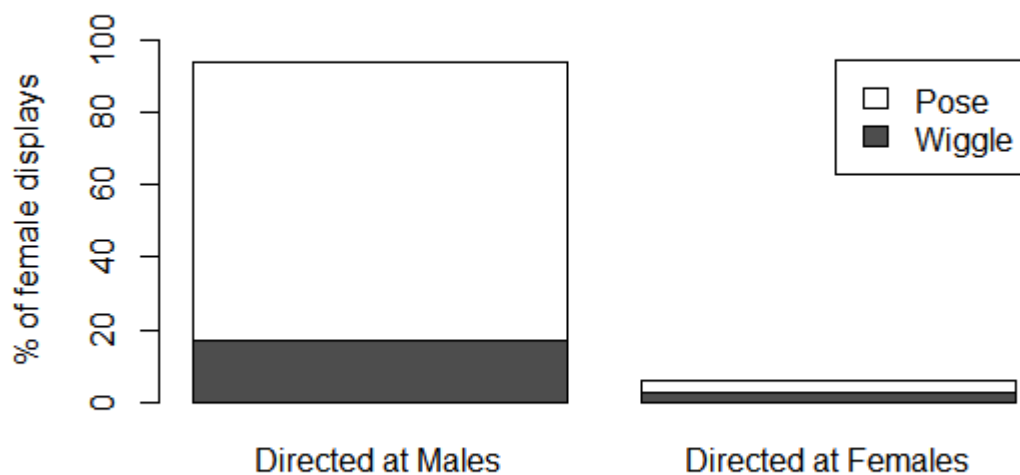
Figure 4 Percentage of active female displays (e.g., poses, in white, and wiggles in dark grey) directed towards males and females.

Group size did not affect the average duration of male wiggles (F-value$_{(3,\ 1414)}$ = 1.03, p = 0.38). The post hoc test also found no significant pairwise comparisons between the four levels of the group size factor (Appendix B, Table 10). The random effect (courtship event) explained little variance in the data (0.021) compared to the residual variance (0.26), suggesting that courtship event is only contributing a small amount to the unexpected variation in the dataset.

Sex and day filmed did not significantly predict the proportion time spent displaying active behaviours in each courtship event (Sex: $F_{1,169}$ = 0.41, p=0.52; Day filmed: $F_{6,111.14}$ = 1.32, p=0.25) (Table 5). Time of day did significantly predict the proportion of active behaviours displayed during each courtship event, with an increase of active courtship seen

in the morning samples ($\beta_{(Noon)}$ = -0.038, [95% CI (-0.53, -0.15), post hoc: t ratio$_{(161)}$ = 3.44,

p= 0.0007) (Figure 5).

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Sex	0.121966	0.121966	1	169.0001	0.411528	0.522064
Time of day	3.555804	3.555804	1	160.9567	11.99768	**0.000683**
Day filmed	2.350289	0.391715	6	111.1397	1.321689	0.253415

Table 5 Anova summary of linear model for the proportion time spent displaying active courtship within each courtship bout.
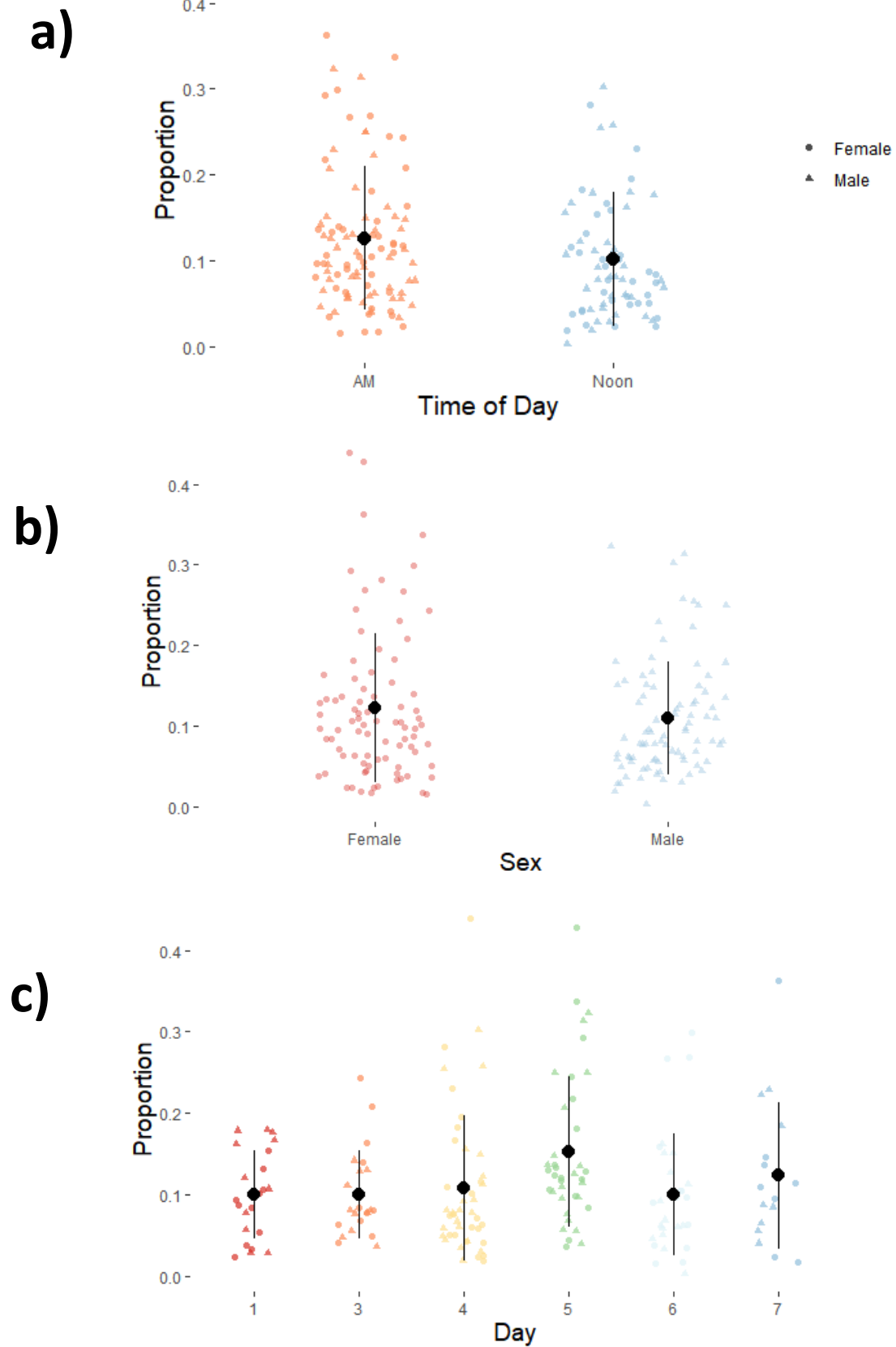
Figure 5 Graphs showing mean (±SE) proportion of active courtship (poses and wiggles) in each courtship bout by a) day filmed, b) sex and c) time of day. Only time of day was found to have a significant difference

Discussion

The aims of this research were to 1) describe the courtship behaviours displayed by *S. nigra,* 2) determine the role of the female ornament in courtship and 3) identify sex differences in courtship displays. I described two key courtship behaviours demonstrated by males and females. Males were observed wiggling, which was always directed towards females. Active female courtship behaviours consisted of poses and wiggles. During both female behaviours the ornament was actively displayed, therefore the ornament plays an important role in the courtship displays of this species. Courtship was most active during the morning. Males initiated courtship events more frequently than females and there was no significant difference in courtship activity between sexes. These patterns were unexpected as I anticipated that females, being the ornamented sex, would instigate courtship and be most active, as seen in other sex-role reversed species such as *Nerophis lumbriciformi* and *Syngnathus typhle* (Berglund & Rosenqvist, 1993; Monteiro et al., 2002). Female fleeing behaviour was also unexpected but frequently observed in our mesocosms. Together, this research suggests that the female ornament in *S. nigra* plays an important role in courtship and is likely to be involved in male mate choice, however males were also active courters and therefore females might also discriminate amongst males.

Males always wiggled, and females predominately posed during courtship displays. Males only displayed towards preferred females, whereas females displayed both wiggles and poses to males and rival females. The courtship displays of *S. nigra* were very similar to the species *Urocampus nanus,* in which females also display the belly in a similar position during the lateral display (pose) (Appendix A) (Sogabe et al., 2012). However, in this species both male and females performed lateral displays, whilst in *S. nigra* it was only the females. Flickering, shaking, and quivering courtship behaviours have been displayed by both males and female in other pipefish species (Kornienko, 2001; Monteiro et al., 2006; Silva et al., 2006), showing that other pipefish also have an active behaviour component to courtship, like the wiggle behaviour demonstrated by *S. nigra*. Interestingly *S. nigra* did not display any form of raising up in the water column, a behaviour noted in other syngnathid species (Sogabe et al., 2012; Vincent et al., 1994). Instead, *S. nigra* were often seen courting on the

bottom of the tank or attached to fake seagrass plants. This may be a result of their natural environment, where intertidal conditions may prevent such behaviours, consistent with the explanation proposed by Monteiro et al. (2006) for the species *N. lumbriciformis*. Alternatively, this behaviour may have been limited due to the height of our tanks (387mm in height). *S. nigra* courted more actively in the morning than in the afternoon. Time of day is an important element to Syngnathid courtship with several other studies finding most courtship occurring in the morning period (Masonjones & Lewis, 1996; Matsumoto & Yanagisawa, 2001; Monteiro et al., 2002; Partridge et al., 2013).

Males were selective in mate choice, often seen wiggling towards large, ornamented females, comparable to other pipefish species (Anderson & Jones, 2019; Berglund & Rosenqvist, 1993; Berglund & Rosenqvist, 2001; Berglund et al., 1986; Partridge et al., 2013). However, I could not identify individuals within my mesocosms, and thus cannot confirm whether males consistently courted the same female and if she was indeed larger than other females in the tank. Often multiple males were seen courting the same large female, this may be due to the female being the preferred mate in the tank. Male pipefish from other species have shown preference for large, ornamented females as these traits correlate with female quality and condition (Anderson and Jones 2019; Berglund and Rosenqvist 2001; Bernet et al. 1998; Partridge et al. 2013). Mating with high quality females can have direct benefit on offspring and therefore is beneficial to males (Paczolt & Jones, 2010; Sandvik et al., 2000). An alternative explanation may be that males were displaying mate copying, a phenomenon observed in *S. typhle* (Widemo, 2005). Males were found to copy other males when selecting a female to court (Widemo, 2005), potentially explaining why I observed multiple males courting one female.

I scored 31 female-female interactions in which a female actively displayed their ornament to the female recipient. These displays are most likely a case of intrasexual competition. It should be noted that female-female competition events were often observed as two females displaying towards each other outside of a courtship bout involving males, not within a courtship event as described for several other species (Monteiro et al., 2006; Rosenqvist & Berglund, 2011; Silva et al., 2006). One explanation for

these displays is the possibility that females may have mistaken the sex of their recipient; however homosexual courtship behaviour seems less likely than competition, since female ornaments have been used in female-female competition in other pipefish species (Bernet et al., 1998). It is therefore a possibility that the female ornament has a dual purpose, which has been observed in other female ornaments (Berglund & Rosenqvist, 2008; Markevich, 2020; Thys et al., 2020).

Most of the female courtship observed was displayed by the largest females in the mesocosms, with smaller females not engaging in courtship. This strong dichotomy could be due to social dominance of the larger females causing smaller females to suspend any courtship behaviour (Berglund & Rosenqvist, 2008; Earl et al., 2021; Owens et al., 1994). Suppression of subordinate courtship has been observed in cichlid fish; when a dominant male is present, subdominant males cease courtship and competition behaviours, indicating that social environment might modulate the behaviour of smaller individuals (Desjardins et al., 2012). If this same mechanism is occurring in the wide-bodied pipefish, the ornament is likely experiencing intrasexual selection through competition in addition to the intersexual selection imposed by the male preferences for large, displaying females.

Under sex role reversal, females are predicted to initiate courtship, as they are considered the courting sex, however I found that males initiated more courtship events than females. Although both sexes initiated failed courtship equally. The initiation of courtship differs between species of pipefish, with females initiating courtship in the species *N. lumbriciformis* and *S. typhle* (Monteiro et al., 2002; Berglund & Rosenqvist, 1993), both sexes initiating in *S. abaster* and *S. scovelli* (Partridge et al., 2013; Silva et al., 2006), and males initiating courtship in the species *S. acusimilis* (now *Syngnathus schlegeli*) (Kornienko, 2001) (Appendix A). In the species with male-initiated courtship, no ornamentation or sexual dimorphism has evolved (Kornienko, 2001). I found no difference in female and male courtship activity, with neither sex actively displaying for a larger proportion of the courtship bout than the other. Under sex role reversal, I predicted that female *S. nigra* would be the courter, initiating courtship and being the most active sex, hence displaying for a larger proportion of time (like in other pipefish species Berglund & Rosenqvist, 1993; Monteiro et al. 2002). These unexpected results might indicate that females were

demonstrating mate preference and were hesitant to engage in courtship with undesired males (Berglund et al., 2005; Goncalves et al., 2015).

Choosiness is quality-dependent and can fluctuate depending on the mating market (i.e., who is available), causing mutual mate choice to be expressed (Chevalier et al., 2020; Edward & Chapman, 2011; Schlupp, 2018). If many suitable mates are available, individuals (both male and female) should be selective preferring only the best quality males, even if their typical role is that of a courter. Mutual mate choice of this nature has been described in sex-role reversed pipefish, where female *S. typhle* and *S. abaster* showed a preference for larger males (Berglund et al., 1986; Silva et al., 2007). Additionally, large female *S. abaster* also demonstrated differential egg allocation with larger males receiving more and larger eggs from these females (Silva et al., 2009). Larger male pipefish invest more energy per offspring and carried heavier embryos (Berglund et al., 1986; Goncalves et al., 2015; Flanagan et al., 2017). Therefore, female with preferences for larger males are able to enhance their offspring's survivorship, including in these classic examples of sex-role reversed species with strong sexual selection via male mate choice on female traits (Anderson and Jones 2019; Berglund and Rosenqvist 2001; Partridge et al. 2013) and frequent female initiation of courtship (Berglund & Rosenqvist, 1993; Monteiro et al. 2002).

Despite female *S. nigra* displaying an ornament to attract males, males often appeared to be the aggressor in courtship displays by chasing females around the tank. This chasing behaviour might be a form of male harassment, which is unexpected in sex role reversed systems. In species such as *N. ophidion, S. typhle* and *S. abaster* there is an absence of overtly aggressive behaviours in either of the two sexes (Silva et al., 2006; Vincent et al., 1994). However, in *Corythoichthys haematopterus* (Matsumoto & Yanagisawa, 2001), *Syngnathus schlegeli* (Watanabe et al., 2000) and *N. lumbriciformis* (Monteiro et al., 2002), females are observed disrupting the courtship ritual of the courting pair. In *C. haematopterus* and *S. schlegeli* female interruptions would occur during the parallel swim phase of the courtship ritual (Matsumoto & Yanagisawa, 2001; Watanabe et al., 2000), which resembles the chasing behaviour observed in *S. nigra*. However, in *C. haematopterus* and *S. schlegeli* groups only consisted of multiple females competing against each other for one male (Matsumoto & Yanagisawa, 2001; Watanabe et al., 2000), unlike *S. nigra* where

multiple males would chase one female. Some bouts of chasing were extremely long (e.g., 1-2 minutes), and are likely energetically demanding on females. Male harassment of this nature can impact female fitness costs (Bacon & Barbosa, 2020; Iglesias-Carrasco et al., 2019) and can alter female movement and dispersal (Severns & Breed, 2018), result in sexual conflict. In my study, this behaviour might have been amplified by the high densities and small tank sizes. For future mesocosm experiments a larger tank may be more suited, with potentially fewer individuals, to reduce the stress associated with the inability to escape persistent male harassment.

My surprising results, male initiation and lack of sex difference in courtship activity, might have resulted from the experimental design. All mesocosms were started with an even operational sex ratio (OSR): eight females and eight males were placed together in each tank, all of whom were sexually mature and reproductively receptive at the start of the trial (i.e., all males had given birth and had empty pouches). However, when collecting pipefish in Tauranga most of the males collected were pregnant, suggesting that natural breeding populations are likely to have a female biased OSR. Our design thus used mesocosms that were more male biased than usual in the wild, a factor that could have altered the courtship behaviour of females and males. Courtship behaviour such as mate choice can be altered with changes in OSR from female to male biased, which can occur naturally throughout the breeding season (Amundsen, 2018; Chevalier et al., 2020; Clark & Grant, 2010; Cunha et al., 2015; Forsgren et al., 2004; Hare & Simmons, 2021; Muñoz-Arroyo et al., 2020; Oyama et al., 2020; Silva et al., 2010; Villarreal et al., 2018; Vincent et al., 1994; Wacker et al., 2014). For example, early in the breeding season, female combtooth blennies (*Meiacanthus kamoharai*) are restricted by the number of available males, causing females to increase the frequency of courtship displays (Oyama et al., 2020). As the females mated throughout the season, reproductive females became limited, and male courtship increased (Oyama et al., 2020). Mate choice can also fluctuate with alterations in mate availability. Male Jamaican field crickets (*Gryllus assimilis*) from female-biased environments showed more mate hesitancy compared to males from male-biased conditions, indicating plasticity in mate choice when the selection of mates was altered (Villarrea et al., 2018). Fluctuations in OSR can also alter levels of intrasexual competition as seen in the sex role

reversed pipefish species *S. abaster*. Males were found to display their ornament in intrasexual competition when OSR was male-biased, when normally no intrasexual competition between males was observed (Silvia et al., 2010). On the contrary, female *S. abaster* did not increase intrasexual competition in female-biased treatments, likely because larger females are preferred and do not need to compete against smaller females (Silvia et al., 2010). Changes in activity and mate choice highlight the plasticity of courtship behaviour under different social environments, indicating that courtship behaviour is dynamic and can show acute shifts.

Overall, the main findings of this study were 1) the female ornament is likely under sexual selection, playing an active role in courtship and possibly intrasexual competition, and 2) males initiated more courtship, and 3) males and females were equally active in courtship. Sexual selection is the likely explanation for the evolution of this novel female ornament, although females also discriminated among courting males. The extent of mutual mate choice observed in this study could have resulted from the social environment of the mesocosms. These results suggest that behaviour can be dynamic and respond to changes in the social environment that occur naturally throughout the breeding season. Future work should test the impact of operational sex ratios on courtship behaviours, whilst also determining whether natural fluctuations occur in wild populations within or between breeding seasons. The female ornament was actively displayed in both courtship and intrasexual displays, however it is still unknown which element or interacting parts of the ornament are under the strongest selection pressures. Furthermore, it is still unknown whether the ornament is an honest signal or results in a trade-off in New Zealand populations and how this might vary geographically. This research has scratched the surface in what we can learn from this sex role reversed species and opens ample possibilities to approach future research questions.

Chapter 3: Variation between populations and honesty of the female ornament

Abstract

In the past female ornaments were assumed to result in a trade off with fecundity, therefore acting as a constraint on the female. Contrary to this assumption, several studies have indicated that females can display honest signals without a fecundity cost. However, ornaments can vary between populations, raising the question as to whether the signalling qualities are maintained across space or whether honesty of female ornaments will also vary across populations. The wide-bodied pipefish, *Stigmatopora nigra*, is a species with an elaborate female ornament and a wide geographic range throughout New Zealand and Australia, making *S. nigra* ideal for investigating this research gap. Using samples from five sites around New Zealand, I quantified the female ornament and body size to determine whether variation was observed between sites. Samples from three of the sites were dissected to investigate whether the female ornament was an honest signal of fecundity. I found that the female ornament and body size varied between sites, with differences observed between the North Island and South Island populations. The female ornament and body size were honest signals of fecundity, with larger, more ornamented females predicted to have a higher fecundity. Female ornaments were honest across population despite the variation found between sites. Overall, female *S. nigra* do not face a trade-off with fecundity when expressing ornaments, rather the ornament acts as an honest signal, and is therefore likely favoured in male mate choice.

Introduction

Variation in ornamentation can exist between populations arising through a multitude of factors (Allen & Miller, 2017; Toubiana & Khila, 2019; Weiss & Brower, 2021). For example, variation can occur through local adaption, condition dependence, genetic drift, and differences in life-history (Emlen, 1994; Kervinen et al., 2015; Svensson et al., 2018; Uyeda et al., 2009). It is important to note that these pathways leading to ornament variation are not mutually exclusive and often interact together to create variation between sites (Slevin et al., 2019). Altered sexual signals can lead to changes in mate preference shifting the selection pressure onto different traits (Dunn et al., 2010). With time this may result in reproductive isolation between populations and eventually even speciation (Ritchie, 2007).

Condition dependent ornaments, as the name suggests, are dependent on the condition of the individual, therefore ornament quality is not inherited, or at least not entirely. For example, in a cross-fostering experiment using house sparrows, badge sizes of sons resembled those of the foster father rather than the biological father (Griffith et al., 1999). Likewise in the horned beetle, *Onthophagus acuminatus*, horn length was not predicted by the size of the fathers' horn, instead the length of this sexual trait was affected significantly by larval food abundance (Emlen, 1994). Therefore, the quality of ornamental traits is not inherited but rather influenced by environmental factors such as nutrition.

Environmental factors can encompass a range of pressures, including climate (i.e., weather patterns), food abundance, and disease, which can impact the expression of an ornament (Weiss & Brower, 2021). Rain and temperature can have a strong effect on ornament expression in several bird species (Moreno et al., 2019), however results were also age and sex-dependent in some cases (Ward et al., 2021; Welklin et al., 2021). Changes in bird ornaments are thought to correlate with fluctuations in insect populations, a food source for many birds, which varies with weather conditions (Moreno et al., 2019; Ward et al., 2021). Other environmental factors such as disease and parasites can also vary between populations. Parasites can decrease body condition of an individual, negatively effecting condition dependent ornaments (Hernández et al., 2021; Kopena et al., 2020). When

parasite numbers fluctuate between populations or when additional disease or infections are present, a reduction in ornament colour saturation (Llanos-Garrido et al., 2017), or structural change (Møller, 2002) can occur. As a result, ornaments can vary across populations, fluctuating with changing environmental factors, leading to variation in ornament expression.

When environmental variation persists between populations, local adaption can occur, shaping sexual ornaments and providing variation between populations (Fargevieille et al., 2017; Svensson et al., 2018). Local adaption to a new habitat, food source, or environmental conditions can cause a change in mating behaviour and sexual selection, which influences how the ornament is expressed (Boisseau et al., 2020; del Sol et al., 2021; Winkler et al., 2012). For example, Japanese rhinoceros beetles (*Trypoxylus dichotomus*) populating two offshore island habitats were found to have shorter horns compared to the mainland sites (del Sol et al., 2021). The new island habitats had an abundance of feeding territories available, which relaxed the selection pressure on males to fight and defend territories, resulting in shorter horn length (del Sol et al., 2021). Over time, local adaptation can alter the strength of sexual selection, causing permanent changes to ornaments (Svensson et al., 2018).

Trait variation between populations can arise without direct selection on the trait. Genetic drift can randomly contribute to variation in traits and mate preferences between populations, causing sexual selection to vary spatially (Tschol et al., 2021; Uyeda et al., 2009). Drift might also interact with genetic architectures, plasticity, and life histories to drive population differentiation. For example, phenotypic variation did not align with patterns of genetic divergence consistent with either drift or selection in populations of Gulf pipefish (*S. scovelli*), suggesting that other factors such as age structure or plasticity drive trait variation (Flanagan et al., 2016). Similarly, genetic differences could not explain the morphological variation in signals of the mormyrid fish, *Paramormyrops kingsleyae*, despite drift being observed between populations (Picq et al., 2020). Variation in sexual traits can also result from differences in life-history, such as lifespan. For example, in the black grouse long-lived males have a lower trait expression for the first 3 years compared to males with a shorter life span (Kervinen et al., 2015). Ornaments may help individuals find and attract a

mate faster, therefore benefiting short lived species which are restricted in the time required to reproduce (Hunter & Bussière, 2019).

Understanding whether variation exists between populations is important in determining how ornamentation has evolved and the plasticity of traits. More generally, variation between populations might explain differences in mating systems, mate preferences, and eventually reproductive isolation and speciation (Mobley & Jones, 2007). For example, in the study by Dunn et al. (2010), female choice differed between two populations of common yellow throats (*Geothlypis trich*). In the Wisconsin population, females preferred males displaying colouration in the form of a black facial mask, which was more variable in this population (Dunn et al., 2010). Females in New York selected the yellow bib ornament, which tended to be brighter and more varied at the New York site than in Wisconsin (Dunn et al., 2010). Both ornaments signal similar aspects of male quality, even though the underlying pigments producing the ornaments differ (Dunn et al., 2010). The similar signalling functions of these two ornaments leads to the question of why ornamental traits vary across populations when they convey the same signalling information. To approach this question, we must first determine whether the ornamental trait is in fact an honest signal, and if so, whether it is honest across populations.

To determine whether traits are honest, other correlating indicators need to be addressed, such as body size and positive allometry. Body size is often related to female fecundity, with larger females producing larger and/or more eggs (Danielsen et al., 2019; Domínguez-Castanedo et al., 2021; Flanagan et al., 2017; Marshall et al., 2013). Hence males favour large over small females as mating partners (Anderson and Jones, 2019; Berglund, 2000). Ornaments can facilitate male preference for large females by amplifying the size difference between females (Berglund, 2000). Larger individuals are often in greater condition and can develop disproportionally larger ornaments showing positive allometry (Summers & Ord, 2021). For example, positive allometry was found for two female ornaments in the dance fly species *Rhamphomyia longicauda*, hence these exaggerated traits highlight the differences among females and serve as a reliable cue to males, as only

larger (presumably higher condition) females can invest in ornamentation (Browne & Gwynne, 2022). Ornaments along with body size can therefore signal female condition during mate choice.

Some female ornaments can be costly to maintain, resulting in a trade-off with fecundity, which has been a central concept to female sexual selection (Fitzpatrick et al., 1995; Irschick et al., 2007). This has been demonstrated in, for example, female fence lizards, where ornamented females have reduced egg mass and a later hatching time compared to non-ornamented females (Swierk & Langkilde, 2013). However, ornamented females had faster sprinting speeds and produced offspring with traits favoured in predator escape and survival (Assis et al., 2018), so there may be benefits as well. Female common yellowthroats (*Geothlypis trichas*) with brighter bib colouration had smaller clutch sizes and higher total brood loss, however bib size was positively correlated with clutch size (Freeman-Gallant et al., 2014). Female ornaments can therefore come at a cost to some aspects of reproductive success, but this might be compensated by other benefits of having the ornament.

Female ornamentation may not be as costly as originally thought (reviewed in (Doutrelant et al., 2020), and in many cases female ornaments are honest indicators of female fecundity (Cunha et al., 2017; Hopkins et al., 2021; Kopena et al., 2020; Lüdtke & Foerster, 2019; Méndez-Janovitz & Macías Garcia, 2017; Mobley et al., 2018; Potti et al., 2013). Female alpine newts bearing an orange ornament were found to lay more eggs and did so faster than less ornamented females (Lüdtke & Foerster, 2019). In female dance flies, pinnate leg scales honestly reflected female fecundity, as females produced larger and more mature eggs when displaying this ornamental trait (LeBas et al., 2003). Mobley et al. (2018) found no evidence that female ornaments, body width and stripe thickness, affected female fecundity in *Stigmatopora nigra*. Larger females maintained larger ornaments resulting in higher fecundity, thus ornaments honestly advertised reproductive fitness (Mobley et al., 2018). Similarly in the black striped pipefish, stripe width was found to correlate with oocyte size, and stripe luminance reflected size of offspring at birth (Cunha et al., 2017). Female ornaments can be honest signals of fecundity as the studies highlight above, but whether there is consistency in honest ornaments across populations is still a gap in the field.

In this study, I will investigate whether a female ornament is consistently honest across a geographic range. My research focuses on the wide-bodied pipefish, *Stigmatopora nigra*, a species with a highly sexually dimorphic female ornament and a wide geographic range throughout New Zealand and Australia. Firstly, I aim to determine whether there is geographic variation in the female ornament across different populations. Secondly, I will determine whether the female ornament is in fact an honest signal. Once I have a basic understanding whether there is variation between populations and if the ornament honestly signals fecundity, I can start to explore whether traits convey the same information across all populations. This final step might not be fully explored in this chapter but will provide insights and options for future research. A similar study on *S. nigra* has been conducted by Mobley et al. (2018) in Australia, which will make for interesting comparisons with the New Zealand populations.

Methods

Samples and sites

Adult female *S. nigra* were collected using a hand pulled seine net. Samples were collected from six locations around New Zealand, across three years (November 2019, November 2020 and April 2021 and December 2021). The most northern locations were Snells Beach (-36.420929, 174.734007) and St Heliers Beach (-36.848657, 174.856085) situated around Auckland (both collected Nov 2019). Plummers Point (-37.66173, 176.04525) and Matua (-37.66255, 176.10827) were two sites in Tauranga (collected November 2020 and April 2021). In the South Island, samples were collected in December 2021 from Otakou (-45.799771, 170.710494) in Dunedin Harbour and Bluff (-46.587291, 168.321894) (Figure 6a). In total there was 21 female samples from Tauranga, 8 from St Heliers Beach, 10 from Snells Beach, 8 from Otakou and 20 from Bluff.

Morphological measurements

Pipefish were euthanized in a dilution of clove oil and seawater (diluted to 100mg/L). Female pipefish were then photographed on a small portable whiteboard with a 15cm ruler attached for scale. Pipefish from Snells Beach, St Heliers Beach and Otakou were measured

out in the field, whilst Tauranga and Bluff pipefish were measured in the laboratory, as they were first used in mesocosm behavioural experiments. The ventral side, with ornament exposed, and dorsal side were both photographed. Only the ventral photograph was measured. Measurements were made from the photographs using the image analysis software ImageJ (Schneider et al., 2012) (Figure 6b). For consistency with the only other morphological study on *S. nigra* (Mobley et al., 2018), I also used snout-vent length (SVL) as a measure of body size. Snout-vent length was measured from the tip of the rostrum to the anal pore. SVL and total body length were highly correlated (r= 0.97, $t_{1,64}$=30.2, p= <0.001), so only SVL was included in downstream analyses (see below). To quantify the female ornament, belly width, band width, and the area of colour between dark bands were measured (Figure 6b). Belly width was measured as the widest point of the belly perpendicular to the SVL, following Mobley et al. (2018). Band width was measured on the dark bands at the midpoint of the SVL along the anterior-posterior axis. Only the first six bands were measured, and the average taken to remain consistent with Mobley et al. (2018). Mobley et al. (2018) used these two measurements to quantify the female ornament, whereas I added the additional measurement of coloured area, which included the entire area between dark bands. Starting after the third dark band, I measured the coloured area between the dark bands for ten bands, using the freehand tool on ImageJ (Schneider et al., 2012). I chose to start after the third band as some the photographs were poor quality and the first two light bands were difficult to identify. This method also allowed the measurements to take place over the belly region of the female where the ornament is most striking. Once females had been photographed, they were stored in tubes of 95% ethanol.

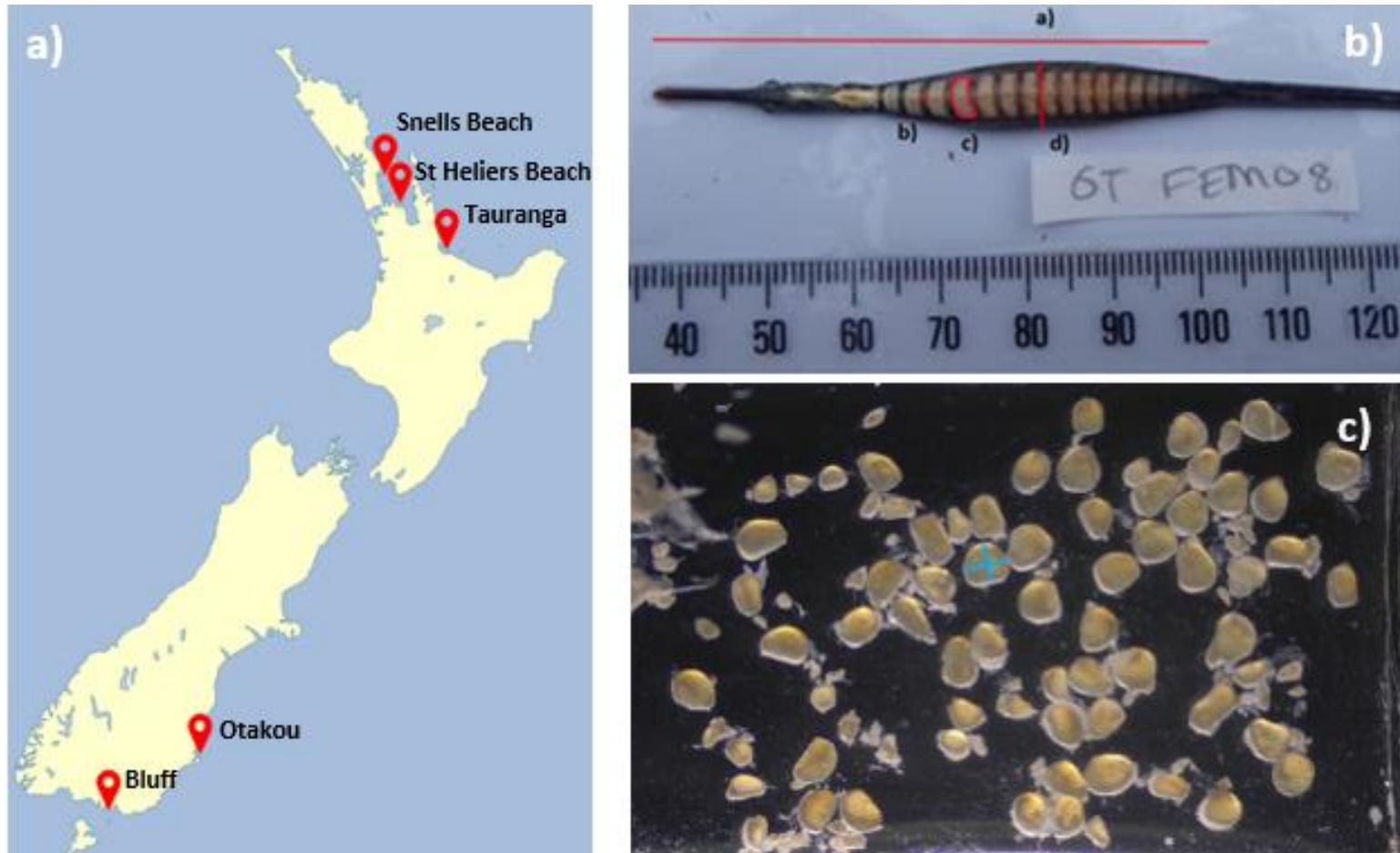*Figure 6. a) Map of five sample sites, b) female S. nigra with ornament measurements: a) SVL (snout-vent length), b) band width, c) area of colour and d) belly width and c) image of mature eggs and blue lines indicating the two diameter measurments discribed in the methods.*

Fecundity measurements

Females from Snells Beach, St Heliers beach, Otakou and Bluff were dissected under a dissection microscope. Using small dissection scissors, a cut was made from the anal pore to a point between the gills. With tweezers, the body cavity was opened along the split to reveal the ovaries. The ovaries were gently removed and placed into custom made slides containing a small walled compartment. Ovaries were covered with distilled water to prevent desiccation. Under a Zeiss Stemi 305 stereo microscope, eggs were gently separated from the ovarian tissue using two needle tools. Once all mature eggs had been separated a photograph was taken using the built-in microscope camera (Figure 6c), immediately followed by a photograph of a ruler for scale. Eggs were considered mature if they were large (at least 10x more than immature eggs), hardened by the ethanol, and yellow in colour, whilst immature eggs were small, remained soft even after preserving, and were white in colour. Contents of the slide were transferred to a pre-weighed aluminium dish and placed in a drying oven set at 50°C for at least 48 hr. Once dry, gonads were weighted on a Sartorius CP225D 5 decimal place scale to obtain the gonad dry weight. Photographs were analysed using ImageJ (Schneider et al., 2012). For each female, 10 mature eggs were measured (like Cunha et al., 2017) by taking two perpendicular linear measures of diameter, to account for the mature eggs' non-uniform shape, and the average was calculated to estimate the mean egg size as done by Mobley et al. (2018)(Figure 6c). The number of mature eggs were counted using the multipoint tool in ImageJ.

Statistical analysis

All analyses were performed in R version 4.0.5 (R Core Team, 2021). To characterize variation in the female ornament I used 18 females from Bluff, 8 from Otakou, 10 from Snells Beach, 8 from St Heliers Beach and 21 from Tauranga. All ornamental traits were correlated with SVL (see results), so traits were standardized for body size by using the raw residuals from ordinary least squares regressions of each trait and SVL (like Mobley et al., 2018). I ran a redundancy analysis (RDA) with site as the constraining variable and standardised ornament measurements (belly width, band width and area of colour) and SVL as the response variables (vegan package v 2.5-7) (Oksanen et al., 2020). Biplots were created to observe the data and ordiellipses using standard errors of means were used to identify clustering in the data.
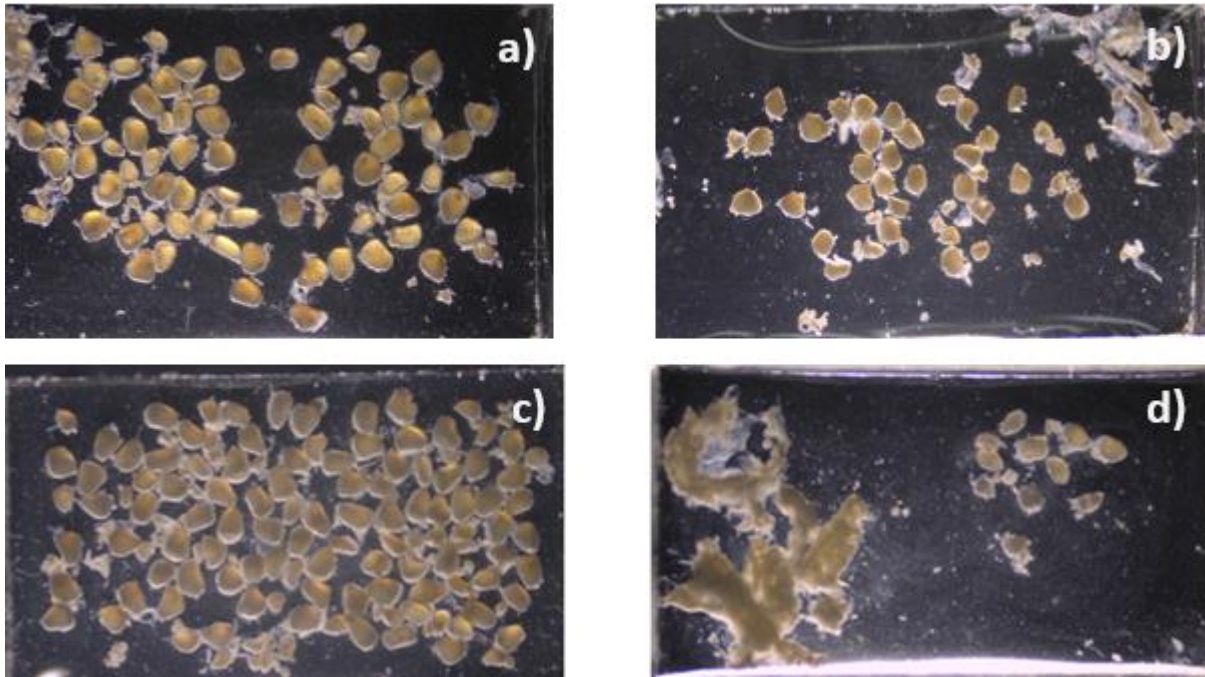
Figure 7 Egg variation across populations, a) St Heliers Beach, b) Snells Beach, c) Otakou and d) Bluff. For all locations except for Bluff photos show the number of eggs in one ovary whereas the photo for Bluff contains both ovaries.

I chose to exclude the 23 Bluff females from my fecundity analysis, as most females had immature eggs in their ovaries, resulting in a very small number of mature eggs (see Figure 7). Two females from St Heliers beach were also excluded from the data as they were sexually immature (i.e., contained no mature eggs in their ovaries).

A Principal Component Analysis (PCA) (vegan package v 2.5-7) (Oksanen et al., 2020) was performed with the three standardised ornament measures, and the site scores from this analysis were used as a multivariate measurement of the female ornament in downstream analyses. This ornament composite, along with SVL and location, were my three predictor variables in my analysis of fecundity, with the number of eggs, size of eggs, and gonad dry weight as response variables. To determine the relationship between fecundity and predictor variables, eight linear models (lmerTest package, R version 4.0.5) (Kuznetsova et al., 2017) for each fecundity measure underwent a model selection process. To determine whether year should be included in the model as a random effect, I examined the residual variance between the two years using the methods outlined in (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). Since the residual boxplots overlapped the zero line and

no pattern was observed, I chose not to include year as a random effect. Two outliers were identified and removed: SH019 was removed from the average diameter model data set and OT04 was removed from the gonad weight model. The number of eggs and gonad dry weight were log-transformed to meet model assumptions. Akaike Information Criterion (AICc) corrected for small sample size was used to select the best fitting model (AICcmodavg package v 2.3-1) (Mazerolle, 2020). If the ΔAICc was < 10, then models were considered to have similar support, and the simplest model was selected.

Results

SVL correlated with all three ornament measures (SVL vs Belly width (r= 0.88, $t_{1,64}$= 14.7, p= <0.0001; SVL vs Band width r= 0.80, $t_{1,64}$= 10.8, p= <0.0001; SVL vs Coloured area r= 0.32, $t_{1,64}$= 2.7, p= 0.008). The summary statistics indicated that female body length, ornament measures and fecundity measures have a wide range (Table 6). At first glance, when comparing my measurements with Mobley et al. (2018) some measurements appear different, however statistical testing is required to make such claims (Table 6).

Population ornament variation

The total variation explained by the RDA constraining variable, site, was 56%. The proportion explained for the unconstrained variables was 44%, with 31% explained by the PC1 axis, 8.6% by PC2, and 3.4% by PC3 respectively. For the RDA1, RDA4 and PC1 axes, SVL and colour had the highest loading (Table 7), whereas for RDA2, RDA3 and PC2, belly width and band size had the highest loading (Table 7). The standard error ellipses show that the South Island sites (Bluff and Otakou) are not clustered with the North Island sites, indicating differences in ornament and SVL between these two locations (Figure 8.a). Tauranga and Snells Beach have overlapping ellipses showing no differences between these two sites (Figure 8.a). St Heliers Beach, however, appears to be clustered separately from Snells Beach and Tauranga, suggesting that there may be differences between these Northern populations (Figure 8.a). Figure 8.b represents the unconstrained variance, hence the variance was explained by other factor excluding site which results in the lack of clustering of sites observed in the plot.
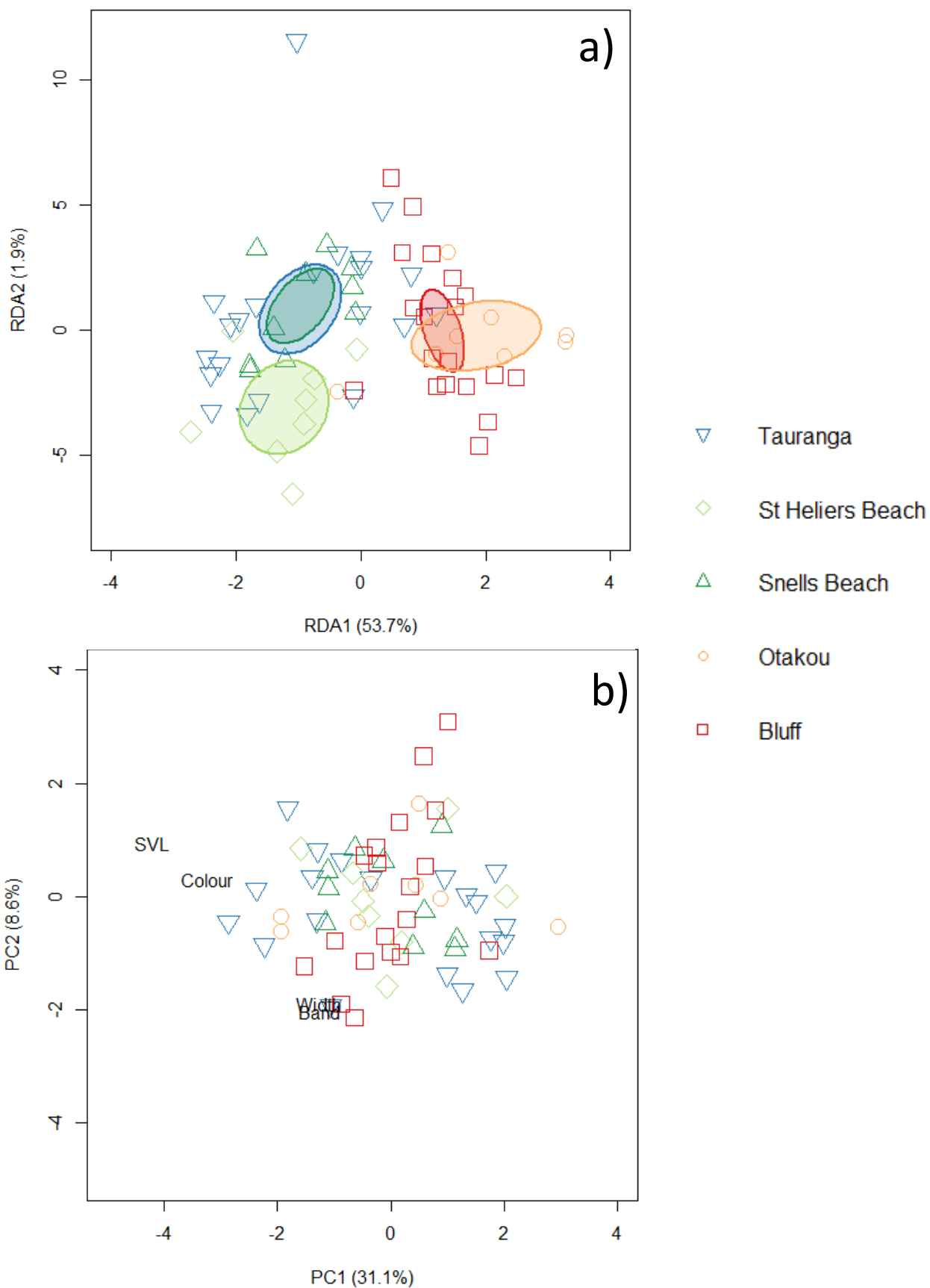
Figure 8 a) biplot showing RDA1 and PC1 axes, b) biplot showing RDA1 and PC2 axes. Ellipses represent clustering of locations, the less the ellipses overlap the more dissimilar the sites

	Gonad weight	Number of eggs	Egg size	SVL	Body Width	Band width	Coloured area
This study	0.013± 0.008	146 ± 58.6	0.76 ± 0.12	47.9 ± 8.0	5.22 ± 1.5	0.79 ± 0.19	2.79 ± 0.5
Mobley et al., 2018	NA	32.9 ± 1.4	0.92 ± 0.01	44.7 ± 0.06	4.53 ± 0.10	0.76 ± 0.01	NA

Table 6 Comparison on my measurements (New Zealand S. nigra) and Mobley et al. 2018 (Australian S. nigra) measurements

	RDA1	RDA2	RDA3	RDA4	PC1	PC2
SVL	4.623335	0.58748	-0.01411	-0.2245	-4.19742	0.943223
Belly width	1.387516	-0.63817	0.449976	-0.05415	-1.27508	-1.88489
Band thickness	2.435697	-1.05928	-0.27573	-0.05644	-1.25618	-2.03649
Coloured area	4.996396	0.149994	0.022514	0.250292	-3.2477	0.308675

Table 7 Species scores for each measure of ornament and SVL across the different axes

Honesty of S. nigra ornament

The next-best model was similar in AICc score (less than 10, see Table 8), I am therefore interpreting the most parsimonious model. The best model predicting mean number of eggs included SVL and ornament ($F_{3,20}$ = 20.42, R^2= 0.70, p= < 0.0001). SVL has a significant positive relationship with number of eggs ($F_{(1)}$= 17.55, p=0.0004) (β (SVL) = 0.196, [95% CI 0.138, 0.253]), indicating that larger females had more eggs. Similarly ornament also had a significant and positive relationship with number of eggs ($F_{(1)}$= 38.59, p= <0.0001) (β (Ornament) = 6.63, [95% CI 4.408, 8.845]), showing that females with larger ornaments had more eggs (Figure 9).

It should be noted that the top two models were equally good with a very small difference in AICc values (0.3, see Table 8). Gonad dry weight was best predicted by SVL and ornament ($F_{2,21}$ =21.46, R^2= 0.64, p= < 0.001). SVL was positively and significantly related to gonad dry weight ($F_{(1)}$ = 22.335, p= 0.0001) ($\beta_{(SVL)}$ = 0.29465 [95% CI 0.184, 0.405]), indicating that larger females had heavier gonads (Figure 9). Ornament also showed a positive significant relationship with gonad weight ($F_{(1)}$= 20.592, p=0.0002) ($\beta_{(Ornament)}$ = 9.27 [95% CI 5.02, 13.51]), hence females with larger ornaments had heavier gonads (Figure 9).

All the other models, apart from the null model (AICc was >10), were very similar in AICc scores (less than 5, see Table 8), I am therefore interpreting the most parsimonious model which excludes location and ornament. The best model predicts egg size using only SVL ($F_{2,21}$ = 24.11, R^2= 0.51, p= < 0.0001). SVL was positively and significantly related with average egg diameter ($F_{(1)}$= 24.12, p=<0.0001) (β (SVL) = 0.009, [95% CI 0.005, 0.012]), thus larger females are predicted to have larger eggs (Figure 9).

Model	K	AICc	Delta_AICc
Number of Eggs			
SVL + Ornament	4	**9.51134**	0
SVL + Ornament + Location	6	15.02454	5.513201
SVL + Location	5	27.65007	18.13873
SVL	3	31.63768	22.12634
Ornament + Location	5	34.50474	24.9934
Location	4	34.93001	25.41867
Null	2	35.20548	25.69414
Ornament	3	35.66876	26.15742
Gonad Dry Weight			
SVL + Ornament	4	**40.68427**	0
SVL + Ornament + Location	6	40.98472	0.300452
SVL	3	54.18001	13.49574
SVL + Location	5	54.739	14.05473
Location	4	58.46522	17.78096
Ornament	3	59.38576	18.7015
Ornament + Location	5	59.90265	19.21839
Null	2	61.86756	21.18329
Average Egg Diameter			
SVL	3	**-54.6396**	0
SVL + Ornament + Location	6	-54.1628	0.476881
Ornament	3	-52.8609	1.778717
Location	4	-52.4696	2.170097
SVL + Location	5	-52.0594	2.580218
SVL + Ornament	4	-51.732	2.907655
Ornament + Location	5	-49.9818	4.65783
Null	2	-39.7195	14.9201

Table 8 AICc results from model selection process. Best fitting model in bold
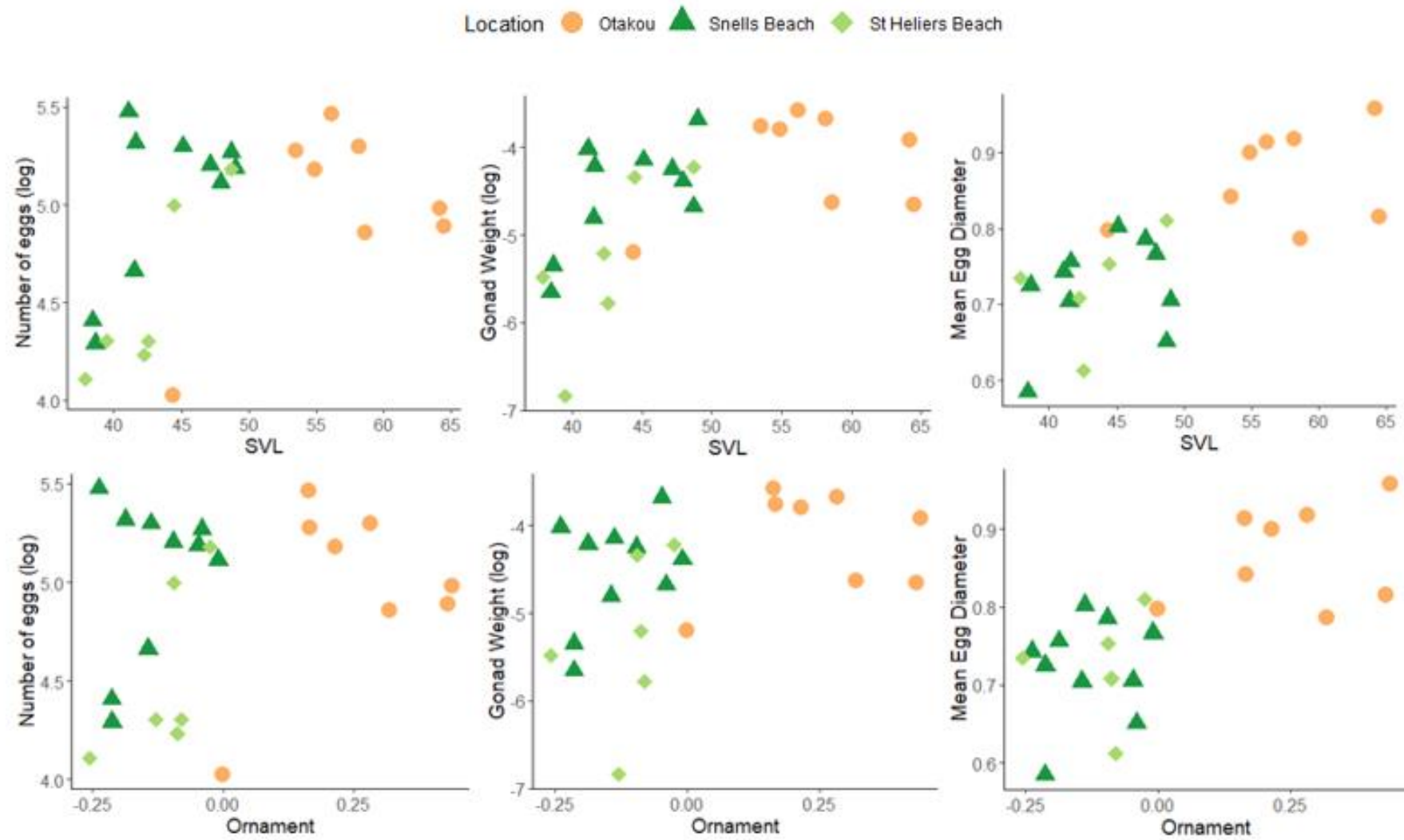
Figure 9 Relationship between the three fecundity measures and the composite ornament metric (top row) and SVL (bottom row).

Discussion

Female ornaments have long been assumed to impose costs on fecundity (Fitzpatrick et al., 1995). In this chapter, I demonstrated significant variation in female *S. nigra* body size and ornamentation across populations in New Zealand. Highly ornamented females were predicted to have larger eggs, heavier eggs, and higher overall number of eggs, suggesting that ornamentation is an honest indicator of fecundity. Despite the variation in ornament and body size, these traits remained an honest signal across populations. Female *S. nigra* therefore do not suffer a trade-off with fecundity when displaying the ornament, demonstrating that despite investing in larger gametes, females can still bear honest signals.

The ornament and body size of female *S. nigra* varied across populations. North Island populations differed from the South Island populations, with both ornamentation and body sizes differing between these two locations (Figure 8a). The proportion of variance attributed to the unconstrained variance could be explained by factors such as age and sexual receptivity (Graves & Eadie, 2020; Kriesell et al., 2021). For example, the size of sexual ornaments correlated with age in great crested newts (*Triturus cristatus*) (Palau Daval et al., 2018). A similar pattern also observed for ornamentation in Golden-winged Warbler (*Vermivora chrysoptera*) (Tisdale et al., 2018). Further investigation is required to determine whether these factors also contribute to trait variation in female *S. nigra*.

Female ornamentation and body size differed between northern and southern populations of *S. nigra*. However, the factors contributing to this variation are unknown. Northern and Southern sites follow a latitudinal gradient (e.g., Snells Beach latitude: -36.420929, Bluff latitude: -46.587291), therefore water temperature is likely to differ between the North Island and South Island populations. In summer, Tauranga water temperature ranges from 18 to 19°C, whereas in Bluff the summer ocean temperatures are around 15°C (Chiswell & Grant, 2018). In other pipefish, population differences in body size have been noted, corresponding with latitude and water temperature. For example, body size in worm pipefish (*Nerophis lumbriciformes*) followed Bergmann's rule, which states that body size and latitude are correlated (Monteiro et al., 2017; Monteiro & Lyons, 2012). Likewise, body size increased with decreasing water temperatures in *S. leptorhynchus*, also

adhering to Bergmann's rule (Wilson, 2009). The Baltic Sea population of broad nose pipefish (*Syngnathus typhle*) showed a decrease in sexual size dimorphism, compared to a well-studied population of the same species found off the Swedish west coast (Cunha et al., 2019), which suggests that latitude is not the only driving factor of population variation in pipefish. Sexual dimorphism and body proportions were also found to differ across populations of *S. typhle*, likely influenced by differing temperature and salinity between populations (Winkler et al., 2012). Body size and sexual dimorphisms can therefore differ across populations, and often are influenced by latitude, which is a possible explanation of the variations found between North Island and South Island populations.

Female *S. nigra* ornamentation also varied between sites and latitude. Likewise, variation in ornaments has been found across latitudes and populations in other species. For example, female facial colouration was found to differ across wide latitudinal gradients in worm pipefish (Monteiro et al., 2017). Similarly, in the Golden-winged Warbler (*Vermivora chrysoptera*), male ornamentation was found to decrease with latitude, as southern birds had lower ornamentation (Tisdale et al., 2018). This pattern was also discovered in insects, with the New Zealand giraffe weevil showing latitudinal variation in body size and weapon allometry (Painting & Holwell, 2014). It is therefore a possibility that latitude is contributing to the variation in female *S. nigra* ornamentation. Alternatively, ornaments can differ due to other factors such as differences in resources (de Sol et al., 2020; Allen and Miller, 2017), habitat shifts (Boisseau et al., 2020), and changes to social selection (Jones et al., 2021; Sirkiä & Qvarnström, 2021; Toubiana & Khila, 2019). Ornament variation can arise through evolutionary mechanisms, and the drivers of trait variation among populations of *S. nigra,* remain unknown. *S. nigra* is found across a wide geographic range (New Zealand and Australia), varying widely in latitude, making it an ideal study species to investigate variation in sexual selection and ornamentation across space.

I found evidence for a positive linear relationship between female ornamentation and fecundity, in that larger more ornamented females were predicted to have a higher fecundity. Mobley et al. (2018) similarly found that female ornament expression correlated to fecundity. SVL and ornament together were found to be best predictors of gonad dry weight and the number of eggs, whereas only SVL was the best predictor of average egg size. Even though my measure of ornamentation was slightly different to Mobley et al.

(2018), a similar trend was observed with width and SVL predicting number of eggs and only SVL predicting egg size. It should be noted that my sample size was considerably smaller than Mobley et al. (2018), due to disruptions in sampling arising from COVID-19 travel restrictions and time limitations. Mobley et al. (2018) also included quadratic relationships into their models which was important in their analysis. I chose not to include these quadratic terms into my models as my smaller sample size was under-powered for this type of analysis. With more samples this should be considered.

Despite these limitations, *S. nigra* show no trade-off between female ornamentation and fecundity, suggesting that the ornament expressed is an honest signal. Likewise in the black stripped pipefish, *Syngnathus abaster*, female ornaments have been shown to signal fecundity, with stripe thickness and truck broadness indicating high fecundity (Cunha et al., 2017). Large female Gulf pipefish (*Syngnathus scovelli*), which tend to be more ornamented than small females, were found to transfer more eggs (Paczolt & Jones, 2010). There is growing evidence that female ornaments are not as costly as previously assumed, with ornamented females showing no trade-off with fecundity in both sex role reversed and conventional species (Hopkins et al., 2015; Hopkins et al., 2021; Lüdtke & Foerster, 2019; Mieno & Karino, 2019; Potti et al., 2013; Weiss, 2006). Instead, female ornaments can honestly signal reproductive quality that provides benefit to males (Cunha et al., 2017; Kopena et al., 2020; Paczolt & Jones, 2010). Ornamented females can not only provide more eggs, but in some pipefish also produce offspring with higher survivorship (Paczolt & Jones, 2010, Cunha et al., 2017). In Iberian green lizards, female coloration predicted several aspects of reproductive investment and success of offspring including hatching success, body size, and condition of offspring (Kopena et al., 2020). Similarly, large female black-finned gobies had higher fecundity and offspring survival, and males preferred larger, wider, and more colourful females (Méndez-Janovitz & Macías Garcia, 2017). Together, these studies provide compelling evidence that males use female ornaments as honest signals of mate quality, and males often prefer large, ornamented females (Cotton et al., 2015; Higham et al., 2021; Hopkins et al., 2015; LaPlante & Delaney, 2020; Lüdtke & Foerster, 2018; Murray et al., 2018; Ng et al., 2017; Partridge et al., 2013; Weiss et al., 2018). It is likely that male *S. nigra* prefer large, ornamented females as these females have a higher fecundity, which benefits males. From these results I can suggest that the female ornament

displayed by *S. nigra* is likely to be under sexual selection, through male mate choice, and honestly reflects female fecundity.

Despite variation in female ornament and body size across New Zealand populations of *S. nigra*, large, ornamented females are predicted to have a higher fecundity in all sampled locations. Through comparing the results of this study with Mobley et al. (2018), it appears that even across larger geographic space signalling does not differ. Hence for *S. nigra* in both New Zealand and Australia the same traits – ornament and body size - are used to honestly signal fecundity. Other ornaments were found to vary between populations but still act as honest signal (de Sol et al., 2020; Dunn et al., 2010). For example, male rhinoceros beetles (*Trypoxylus dichotomus*) varied in horn length across populations, however males with longer horns were still better at competing with other males in all locations (de Sol et al., 2020). Therefore, like S*. nigra,* the sexual trait varied across populations, however horn length remained an honest signal of dominance just as the ornament and body size of *S. nigra* remained an honest signal of fecundity across populations.

Future work on *S. nigra* should increase sample size and include additional populations to gain a better understanding of variation across space and more evidence towards the honesty of the female ornament. Female *S. nigra* display bright colouration which has been observed to range in colour (from yellow to dark orange) across New Zealand and Australia. This colouration has also been observed to change across short time scales (Beasley personal observation). Although I quantified the area in which the colour was found, I did not include the variation of the colour itself. In some species of pipefish female exaggerate colouration during courtship (e.g., *Syngnathus typhle*) (Berglund et al., 1997, Berglund and Rosenqvist, 2001). Colouration is an important element in ornamentation, signalling condition (Kopena et al., 2020, White, 2020; Weiss, 2006), beneficial traits (e.g., sprinting speeds beneficial to predator avoidance, Assis et al., 2018), dominance (Jones et al., 2020; Rigaill et al., 2019), sexual receptibility (Rigaill et al., 2019), and fecundity (Cunha et al., 2017; Meino and Karino, 2019), and is often favoured in mate choice (LaPlante and Delaney, 2020) and intra-sexual competition (Earl et al., 2020). Therefore, the saturation of the *S. nigra* ornament may contribute to signalling fecundity, competition, or body condition. Untangling the mechanisms underlying female colouration

and the selective pressures acting on colour are important directions for future work on this species.

In summary, the female ornament of *S. nigra* is an honest signal of fecundity, as a positive relationship was found between fecundity (e.g., egg size, count and weight) and body size and ornamentation. Variation in body size and ornament was found between five populations around New Zealand, with differences observed between the North Island and South Island sites. Despite variation between populations, the honesty of female ornaments remained constant across populations. Females can therefore maintain honest signals, even across wide geographic ranges, and do not suffer a fecundity trade-off in doing so, contradicting past assumptions of female ornamentation.

Chapter 4: General Discussion

In the past females expressing ornaments were assumed to experience a trade-off with fecundity, reallocating resources away from gamete production towards maintaining sexual traits (Fitzpatrick et al., 1995). Nonetheless, sexual selection can drive the evolution of female ornaments through mate choice and intrasexual competition (Amundsen, 2000a). It is beneficial for males to select a mate using signals that honestly reflect the female's quality, such as body condition or fecundity, traits that increase offspring survival (Tigreros et al., 2014). Examples from a variety of taxa and a range of mating systems do not yield a general pattern, with female ornaments either reducing female fecundity or acting as honest signals, calling for more research in this field (Doutrelant et al., 2020; Swierk & Langkilde, 2013). The wide-bodied pipefish, *Stigmatopora nigra,* have exaggerated female ornaments and, until recently, little was known about the ornament's function and evolution. I found that the ornamentation of *S. nigra* females did not result in a trade-off with fecundity, but rather indicates the reproductive quality of the female, acting as an honest signal. My research also revealed that this striking female ornament is used in courtship displays, and that, against my expectations, males were most frequently the initiator of courtship, aggressively chased females whilst courting groups often consisted of multiple males courting one female, painting a nuanced picture of sex role reversal in this species.

In chapter two, I examined the courtship behaviour of *S. nigra* and found that the ornament was predominantly displayed during courtship towards males. It is therefore likely that the ornament plays an important role in male mate choice. The ornament was also displayed on some occasions towards other females, suggesting that the ornament may also play a role in female-female competition, however since evidence was scarce no solid conclusions can be made. Courtship was most frequent in the morning, although was still observed throughout the day. To my surprise, I found males initiated courtship more often than females, and males and females did not differ in their overall courtship activity, suggesting that mutual mate choice might be more important in this species than previously expected (e.g., Mobley et al., 2018).

In chapter three, I discovered that the female ornament and body size were honest signals of fecundity. The honesty of the female signal remained true across all populations even though variation in ornamentation was found between sites. The female ornament and body size are therefore likely to be used by males during mate choice to identify and select highly fecund females. This hypothesis is somewhat confirmed by the observations from chapter 2 that males were mostly seen courting large, ornamented females, though this was not quantifiable based on the study design and warrants further research. Together, these two chapters suggest that the female ornament is under sexual selection, with mate choice as the driving force. Furthermore, the results suggest that maintaining this sexual trait does not pose a constraint on the female, but rather signals the female's reproductive quality.

Limitations of the research

The next steps in determining whether sexual selection is in fact the key driver of *S. nigra* ornamentation is to further investigate mate choice in this species. This was initially the intention of this research, as I had planned to run several dichotomous choice test trials to determine whether males did in fact prefer larger, ornamented females. This however did not come to fruition due to travel restrictions associated with the COVID-19 pandemic. Results from a very small (5 trials) pilot study that I was able to run after my mesocosm experiment suggested that males did indeed favour large females during mate choice. However, this was only observed when there was a 15mm size difference between the two females in the dichotomous choice test. A similar phenomenon was found in guppies were males preferred larger females and showed a stronger preference for body size as the size difference between the two females increased (Dosen & Montgomerie, 2004). The next step in this research would be to carry out a full-scale experiment investigating male mate choice, to determine whether males are selecting for large, ornamented females, matching the signals that were found to honestly reflect fecundity.

I had also intended to collect more samples for my fecundity experiment from each site as well as adding a few additional sites from the Wellington, Banks Peninsula, and northern South Island regions, but I was again restricted by travel and my master's timeframe constraints. Future work should address these limitations by collecting additional

samples and increasing the number of sites across New Zealand. Despite these limitations, this study gives insight to the *S. nigra* ornamentation, suggesting that the female ornament is likely to be under sexual selection.

Future directions

It is becoming clear that mating systems are more dynamic than traditionally thought, with sexual selection acting on both sexes in different strengths. This was observed in my mesocosm study, as males were found to play an active role in courtship and initiated courtship more frequently than females. Males may therefore be demonstrating mutual mate choice, likely to be expressed when the operational sex ratio (OSR) is less female-biased. The strength of sexual selection has also been found to fluctuate between sexes throughout the breeding season in response to natural shifts in OSR (Amundsen, 2018; Cunha et al., 2015; Hare and Simmons, 2021). Mating behaviours and mate choice are therefore plastic traits, changing in response to the social environment and available mates (Oyama et al., 2020; Silva et al., 2010; Villarreal et al., 2018; Wacker et al., 2014). Natural shifts in OSR in *S. nigra* populations are likely to occur throughout the breeding season, as noted in other pipefish species such as *Syngnathus abaster* (Cunha et al., 2015). This would be an area of further research in which *S. nigra* could be a useful study species. Very little is known about the life-history of *S. nigra* and whether OSR changes throughout the breeding season. Insights from these studies would help frame the plasticity of sex roles and strength of sexual selection, demonstrating how roles are not static within a mating system.

Variation in female ornamentation was observed between sites within New Zealand (Chapter 3), consistent with other studies of ornamentation in a range of taxa (Allen & Miller, 2017; del Sol et al., 2021; Dunn et al., 2010; Fargevieille et al., 2017). Many factors can contribute to the variation observed between populations, many of which interact together and are not mutually exclusive. Environmental conditions such as weather, latitude and resources influence how ornaments expressed (Mário Cunha et al., 2019; del Sol et al., 2021; Monteiro et al., 2017; Moreno et al., 2019; Ward et al., 2021; Welklin et al., 2021). Altered environmental conditions between sites can lead to local adaption, geographic isolation, and genetic drift, all of which can contribute to variation in sexual traits between sites (Boisseau et al., 2020; Tschol et al., 2021). The variation found between populations of

S. nigra can be further explored to determine which factors are contributing to these differences, adding genetic variation alongside trait variation would be an obvious next step. *S. nigra* are an ideal model species for these questions as 1) variation between populations has been identified through this study and 2) they encompass a wide geographic range when including populations around Australia. Variation in sexual traits and altered sexual selection pressures can have further consequences on species divergence, speciation and extinction, causing this field of research to have further implications in a wider evolutionary context.

Implications

Overall, the findings from my research contribute to the wider field of female ornament evolution. Female ornaments can evolve through both intra- and inter-sexual selection, supporting the conclusion that sexual selection theory can act on traits in both sexes (Doutrelant et al., 2020; Hare & Simmons, 2021; Hernández et al., 2021; Hopkins et al., 2015; Mieno & Karino, 2019; Ng et al., 2017). Females expressing ornaments were thought to be disadvantaged in the past, as assumptions presumed there to be a trade off with ornament expression and female fecundity (Fitzpatrick et al., 1995). Although true for a number of species (Assis et al., 2018; Freeman-Gallant et al., 2014; Swierk and Landkilde, 2013), there is also compelling evidence that female ornaments can correlate with female fecundity and other measures of quality (immunity, parasite load and body size), and therefore act as honest signals in mate choice (Kopena et al., 2020; Lüdtke & Foerster, 2019; Lumpkin et al., 2014; Mieno & Karino, 2019; Painting et al., 2017; Weiss, 2006; Weiss et al., 2013; White, 2020). My results support the conclusion that female ornaments can act as honest indicators of fecundity that are used by males in mate choice. The research field of sexual selection on females has made a shift in recent times, with new discoveries that go against the founding viewpoint that females are constrained by ornaments and merely by products of sexual selection acting on males (Amundsen, 2000a, 2000b; Doutrelant et al., 2020; Fritzsche et al., 2021). The shifts in research focus, to examine sexual selection on females, may in part be related to a more diverse range of scientists evaluating the research field, contributing to new questions and approaches in science. For example, female researchers have driven the study of female bird song, which in the past, like female ornaments, were overlooked and understudied, despite playing an important role in mating

behaviour (Haines et al., 2020). Future studies on sexual selection in females will further contribute to the complexities of mating systems, and the interacting factors that determine how ornaments are expressed.

Variation in ornamentation can alter the strength of sexual selection, and thus can lead to speciation and potentially extinction. In our changing world, ornament signalling may be affected, altering sexual selection within a population. Pipefish have already been used to address the possible impacts climate change and anthropogenic effects will have on the evolution of mating systems (see Monteiro et al., 2017; Rose et al., 2013; Sundin et al., 2017; Sundin et al., 2016). This area of research is becoming increasingly more important with respect to how sexual selection will readjust in response to climate change and whether it will drive certain species to local extinction.

In conclusion, I found that the female ornament in *S. nigra* played an important role in courtship, varied across populations and was an honest signal of fecundity across all populations. Variation in ornamentation and sexual selection pressures across populations is an area of research that is becoming increasingly important. As our environment changes, so will selection pressures. Therefore, being able to predict how traits such as ornaments and behaviours interact with the environment is critical to being able to respond to anthropogenic changes. Mate choice decisions are critical components to the ongoing survival of species, contributing to speciation and potential local extinction, highlighting the relevance and wider implications of sexual selection research.

Appendix A

Comparison of courtship behaviours across pipefish species

Species	Ornament	Courtship Behaviours	Which sex initiates courtship?	Which sex is more active in courtship?	Intrasexual competition	Reference
Urocampus nanus, barbed pipefish	Females ornamented (bright blue spots along head and body)	• Lateral display - bend body to display belly to counterpart • Rise up simultaneously in water column • Hold tails whilst lateral displaying • Parallel swim, male and female maintaining close distance	Both sexes	NA	Males intervene courting pair. Female directs courtship behaviour towards particular male.	Sogabe et al., 2012
Syngnathus acusimilis, Primor'e pipefish	No ornamentation or sexual dimorphism	• Males initiate courtship shaking • Male activity attracts females • Females become active • Several males swim parallel to females • Every 10-20 min males shook • Females coiled into rings and straightened out promptly	Males	Males	Males compete with one another for access to females when density of females is low	Kornienko, 2001

Syngnathus abaster, black-striped pipefish	Amplifiable ornament, females larger than males	• Mutual flickering that affects the entire body – bending along the main axis • On flicker caused response in the opposite sex • After each sex flickers they swim together in parallel fashion performing rapid vibrations • Touching each other with tails or flanks • Flicking behaviour increased in frequency and female papilla becomes visible	Both sexes	Both sexes active	Females disrupted other mating pairs, placing body between courting pair	Silva et al., 2006
Nerophis lumbriciformis, worm pipefish	Female ornament – amplifiable (stripped pattern)	• Female approaches male, starts quivering (only effecting body, not head) • Repeated quivering – only female displays this behaviour • Female moves forward slowly – if male follows quivering to crescendo • Female head vibrates in vertical movement	Females	Females	Females disrupt courtship of mating pairs – placing themselves between the quivering pair	Monteiro et al., 2002
Syngnathus typhle	Female ornament – amplifiable (females brighter), females larger than males		Female	Females		Berglund & Rosenqvist, 1993

Syngnathus scovelli	Female ornament – amplifiable (iridescent stripes), females larger than males		Both sexes			Partridge et al., 2013

Appendix B

Pairwise comparisons

*Table 9 All statistically significant pairwise comparisons for pipefish behaviours.*

Comparisons	Z	P-adjusted
Active movement – Chasing	-3.51925795439937	0.0142809386391943
Active movement – Cleaning	5.96144645732925	8.25048976516428e-08
Chasing – Cleaning	6.47561481397528	3.11607954724317e-09
Cleaning – Courtship	-6.86112425769166	2.25458316558439e-10
Active movement – Directional swim	6.43794252198776	3.99642671091955e-09
Chasing – Directional swim	6.38817186927966	5.5400596700184e-09
Courtship – Directional swim	7.28468934562782	1.06396079730918e-11
Active movement – Floating	6.32056735874085	8.59995518660603e-09
Chasing – Floating	6.52795408629176	2.2002461835759e-09
Courtship – Floating	7.19221618348953	2.10368352634913e-11
Cleaning – Interaction	-6.09267340790266	3.66433502257343e-08
Directional swim - Interaction	-6.05786943365404	4.55189733314775e-08
Floating – Interaction	-6.18887385730406	1.9996530809633e-08
Active movement – Non-active movement	-27.0659345235444	8.1848830589363e-160
Chasing – Non-active movement	-5.60257100258655	6.96945078416904e-07
Cleaning – Non-active movement	-21.1747362738587	5.38867969311371e-98
Courtship – Non-active movement	-17.7691064473994	4.04674090312414e-69
Directional swim – Non-active movement	-26.0223129121122	9.13853075466032e-148
Floating – Non-active movement	-23.3115389272245	1.11745452422491e-118
Interaction – Non-active movement	-9.2770553396966	5.7494904808718e-19
Active movement – On plant	3.45102435774325	0.0184292844551559
Chasing – On plant	4.83494714328457	4.39497360393744e-05
Cleaning – On plant	-3.73279052501613	0.00624921254944909
Courtship – On plant	4.82936098017586	4.52007279630609e-05
Directional swim – On plant	-3.62684295356974	0.00946795317795911
Floating – On plant	-3.84781705200623	0.00393277479392111
Interaction – On plant	4.15450073032796	0.001075795301777721
Non-active movement – On plant	29.2698803104127	8.27682173591755e-187
Non-active movement - Spinning	4.5383436084883	0.000187102810334292
Cleaning – Tails	-3.34276351382687	0.0273730238164208
Floating – Tails	-3.2944875521805	0.0325384328582965
Non-active movement – Tails	5.44804065132133	1.68061476719338e-06

Comparisons	estimate	SE	df	t ratio	p value
2 − 3	0.047375	0.033717	1286.694	1.405077	0.496346
2 − 4	0.077599	0.051862	979.1391	1.496245	0.440127
2 − 5	0.049053	0.081335	1383.385	0.6031	0.9311
3 − 4	0.030224	0.049441	1547.676	0.61131	0.92851
3 − 5	0.001678	0.078956	1641.682	0.021254	0.999997
4 − 5	-0.02855	0.080545	1754.263	-0.35441	0.984738

Table 10. Pairwise comparisons of post hoc test for group size linear mixed effects model

Appendix C

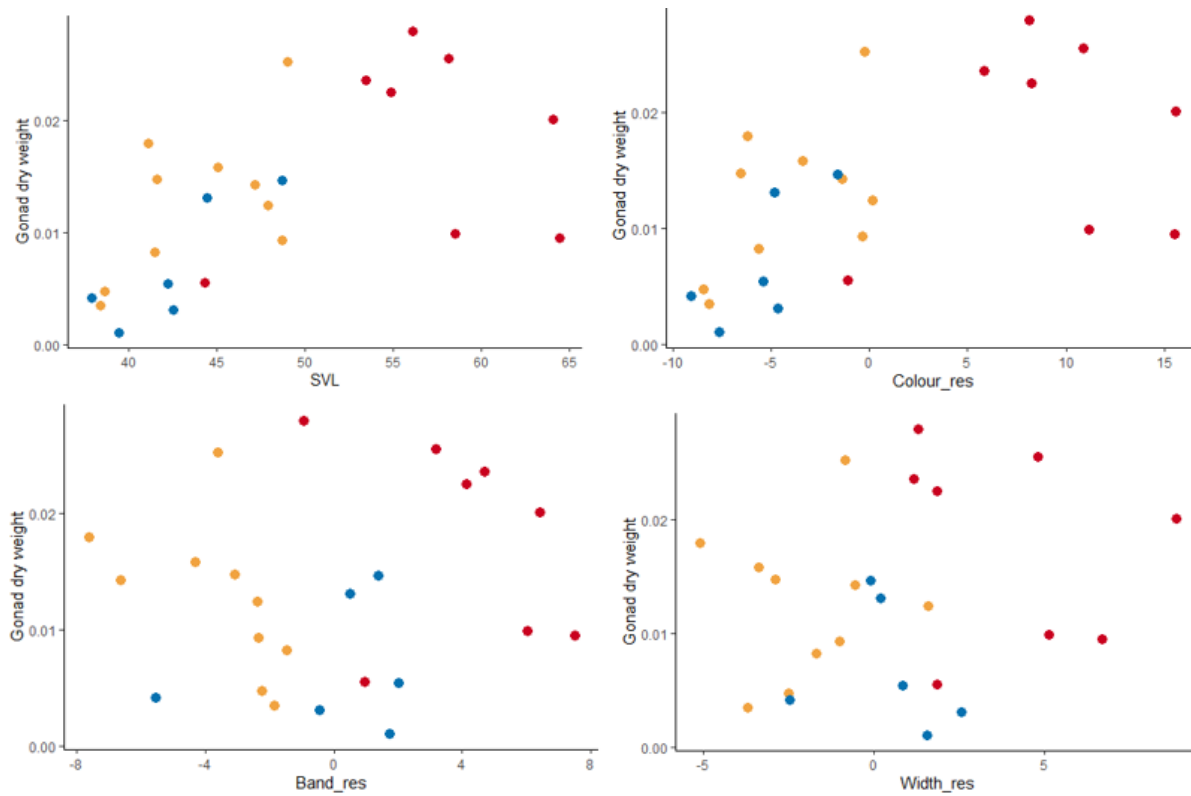## Plots of raw data for each fecundity and ornament measure
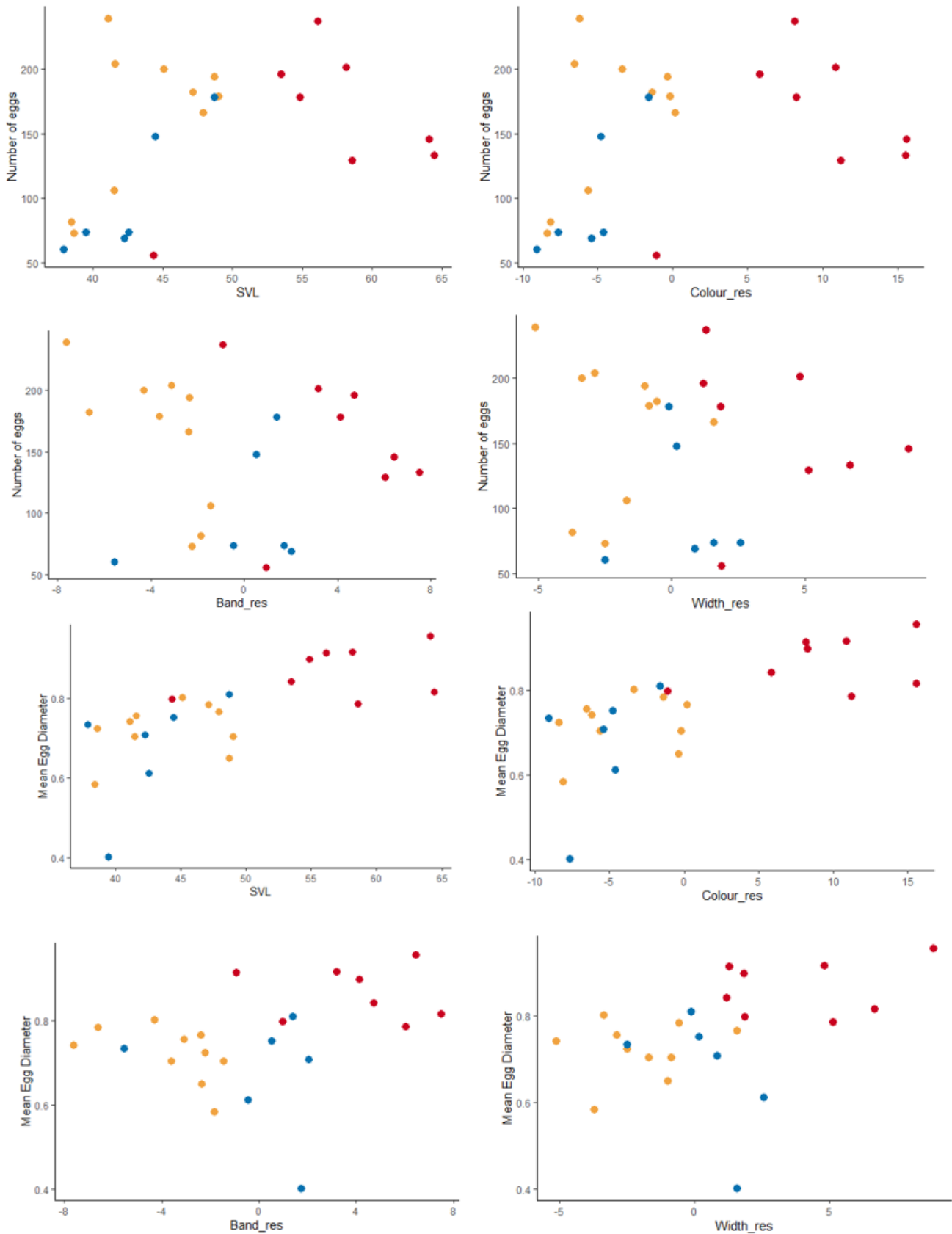
Figure 10. Raw data plots. Each fecundity measure (egg count, egg size and gonad dry weight) plotted against standardized ornament measures (coloured area, band width and belly width) and SVL

Appendix D

Supplementary Material – Pipefish Care and Transport

Transporting pipefish

A total of 10 chilly bins (40L) where used to transport live pipefish from Tauranga to Christchurch in a large van, with each chilly bin containing 25-30 pipefish. Sexes and sites were kept separate during transport. Cooler bins were lined with transparent plastic bags filled with sea water and 5 fake seagrass plants to provide shelter for the pipefish. A bubbler line was placed in each of the coolers to aerate the water during the trip back to Christchurch. The plastic bag was tied shut but loose enough for air to still move freely through the airline. The bubbler was taped to the top of the cooler lid to hold it in place. We used plastic bags minimise water spilling out of the cooler box and avoid vigorous movement which could disturb or harm the pipefish. Two ice packs were placed outside of the plastic bag within the chilly bin to keep the temperature constant. Once underway the temperature was checked every 2-3 hours using a temperature probe to ensure that the water temperature was not increasing. If it increased, extra ice packs and fresh seawater was added to cool the water. All pipefish survived the journey from Tauranga to Christchurch.

Acclimatising Pipefish

On arrival to the laboratory, pipefish were first acclimated to the water temperature of the tanks (also carried out in (Partridge, Boettcher, & Jones, 2013). For this we placed one plastic bag filled with sea water and pipefish into each of the 10 tanks in the aquarium. Once the temperature inside the bags was equal to the temperature in the tanks pipefish were gently released into the tank.

System set-up

Two flow-through systems were set aside in the University of Canterbury aquarium to house the pipefish. Tanks were H 645mm by L 494mm by W 387mm were set up for the

10 trials to take place in. The tanks were made from transparent plastic storage containers that had been modified to fit the aquarium inflow and outflow system. White plastic was taped to either side of the tank to prevent pipefish seeing other pipefish in the adjacent tanks (like in (Anderson & Jones, 2019)). Another 5 opaque tanks were set up on the other system and as holding tanks. The temperature for both systems was set to 18°C which mimics the water temperature in the natural environment. Temperature is an important factor as pipefish will not mate or court without the right temperature cue (Silva et al., 2006a) Seawater cycled out of tanks through biofilters into reservoir tanks, which served as the source of in-flowing water for each pipefish tank. Reservoir tanks were topped up regularly with fresh seawater, which was colder but was heated overtime in the systems. Each tank contained at least 5 fake seagrass plants made from plastic ribbon which had been curled and attached to stones weights. Pipefish used these fake plants for refuge. Lights were set to a 12hr daylight cycle.

Pipefish care and feeding

All tanks containing pipefish are cleaned once daily before the PM feeding. Using a siphon, organic matter was sucked from the bottom of the tanks and into a bucket and disposed of.

Feeding was carried out twice daily. Pipefish were fed artemia, brine shrimp (like other pipefish studies (Anderson & Jones, 2019; Paczolt & Jones, 2010; Partridge et al., 2013; Gunilla Rosenqvist & Johansson, 1995; Sandvik, Rosenqvist, & Berglund, 2000; Sundin, Aronsen, Rosenqvist, & Berglund, 2017)). While pipefish were feeding, inflow tubes were either removed or turned off to stop the system from cycling. This ensured that the small brine shrimp were not cycled out of the tanks before the pipefish could feed. The systems were stopped for an hour allowing pipefish ample time to feed.

Artemia were raised in a temperature control room set to 25°C. Brine shrimp were hatched in plastic cones containing an airline to simulate turbulence. Two cones were set up to be fed out with another one 2L cone and two 1.25L cones set up to resupply the two feeding out cones. Feeding out cones contain 2-day old brine shrimp. In the morning, the AM cone was settled by removing the airline and leaving cone to stand for ~10 minutes

causing brine shrimp to settle on the bottom of the cone whilst the empty egg cases would float to the top. Egg cases are harmful for pipefish digestion thus this step was essential. The cone was then drained through a fine mesh net to catch the brine shrimp. The shrimp were transferred to a beaker containing 500ml of fresh seawater. This created a concentrate that was divided over the tanks of pipefish using a turkey baster. The empty feeding out cone was then washed out with freshwater and vinegar and replenished with the 2L cone containing 1-day old brine shrimp. The empty brine shrimp hatching cone was also washed out with freshwater and vinegar and then reset using the quantities outlined in the table.

Table 11 Quantities of ingredients for brine shrimp rearing

Cone Size	Water (fresh)	Table salt	Epson salt	Brine Shrimp eggs
2L	1.5L	2.5Tbsp	1tsp	¾ tsp
1.25L	800ml	1.5Tbsp	½ tsp	½

The PM feed was carried out using the same methods outlined for the AM feed, except the feeding cone was replenished with two 1.25L hatching cones and these were refilled using the measurements stated in the table. 1-day brine shrimp were also fed 10 drops of Artemio® fluid, a liquid algal solution after being transferred to the empty feeding cones.

Tagging pipefish

The first 5 trials were tagged using Visible Implant Elastomer tags, a tagging system used for previous pipefish studies fish (Flanagan et al., 2014; Flanagan et al., 2017; Rose et al., 2013). Each trial consisted of females and males from both Plummers point and Matua sites. A range of female and male body sizes were included in each mesocosm to represent a natural population. Each pipefish was first sedated using clove oil and seawater dilution. Individuals were placed in the solution until they were unresponsive. Once the pipefish failed to react to touch, pipefish were injected with the elastomer tags on the ventral side of the body using the unique codes provided by Northwest Marine Technology, Inc. After

tagging pipefish were measured and photographed on a small portable white board with a ruler attached to the surface. Males were photographed on all four sides, ventral, dorsal, and left and right sides, whilst females were only measured on the dorsal and ventral sides due body morphology hindering the placement on either side. Once the fish had been tagged, measured and photographed they were replaced into a bucket with an aerator. Pipefish recovered within 15 minutes. Tagged fish were given two days to recover from tagging before the experiment began.

Whilst tagging had been successful in other pipefish studies (Flanagan et al., 2014; Flanagan et al., 2017; Rose et al., 2013) it may have contributed as a stressor to our pipefish, leading so some deaths. Additionally, tags were too small to see and therefore could not help identifying individuals in the video footage, which was the original intention. As result we decided to stop tagging fish for the final 5 trials to prevent more deaths. Tagging was not essential as individuals could be identified using body measurements and unique features. Trials that had lost fish between tagging and the start of the trial were replaced with spare pipefish or individuals from other trials, to prevent delaying the start days of these trials. For the remaining untagged trials fish were measured in a small glass tank with a ruler attached to the side. This reduced the amount of time the fish needed to be out of the water. Individuals were pressed up against the glass gently and body length recorded. Each fish was examined for unique traits such as colour and patterns. This was used to later match individuals to photos.

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