University of Antwerp 2014-2015

Faculty of Science, Department of Biology

Master Project submitted to obtain the degree of Master in Biology, specialisation Evolutionary and Behavioural Biology

Artificial selection in guppies:

male and female phenotypes produced by inter- and intrasexual selection

Jens Van Eeckhoven

Promotor: Dr. Katleen Huyghe[†]

Co-promotor: Prof. Dr. Raoul Van Damme[†]

[†] Research group: Functional Morphology

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1 Abstracts

1.1 English abstract

The effects of inter- and intrasexual selection are often assumed to coincide, yet this is not always the case. The current study used two artificial selection lines to attempt to disentangle the effects of inter- and intrasexual selection in *Poecilia reticulata*. Female choice, male reproductive behaviour, male aggression and male morphology were tested and compared between inter- and intrasexually selected individuals after three generations of artificial selection. It was hypothesised that daughters of each line would prefer males of their own line of selection. Furthermore it was hypothesised that intrasexually selected males would show less colouration, more aggressive behaviour and would attempt to force more copulations than their intersexually selected counterparts. It was found that females overall, preferred intersexually selected males, regardless of their own origin. Intrasexually selected males did not show any more aggression, did not perform more harassment or forced copulation attempts and showed little difference in colouration, but they did have larger tails on average. Intersexually selected males spent more time advertising their colours, yet they did not perform more displays. A significantly higher variance in orange colouration area was found for intersexually selected males, and orange colouration area and patterns are argued to be subject to negative frequency dependent selection by means of female choice. In general these results disconcur with the assumption that intra- and intersexual selection always reinforce the same or similar characters, and show that they can exert differential effects on behaviour (showiness) and morphology (tail size). This is likely due to the lack of direct benefits of choosing a dominant male, who do not defend territories and do not provide resources for their mate.

1.2 Dutch abstract

Seksuele selectie wordt klassiek opgedeeld in: interseksuele selectie (vrouwelijke partnerkeuze) en intraseksuele selectie (strijd tussen mannetjes onderling om wijfjes). Deze twee vormen van seksuele selectie kunnen samen inwerken op gelijkaardige kenmerken (uiterlijke zowel als gedragskenmerken), maar dit is niet altijd het geval. In deze studie probeerde ik kenmerken onder invloed van deze twee types selectie van elkaar te onderscheiden bij zowel mannelijke als vrouwelijke guppies (*Poecilia reticulata*). Dit deed ik aan de hand van twee artificiële selectielijnen die werden onderhouden, die deze twee types van seksuele selectie nabootsten. Vrouwelijke zowel als mannelijke guppies van elke selectielijn werden met elkaar vergeleken. Verschillen in partnerkeuze en reproductieve gedragingen werden vergeleken tussen vrouwtjes. Terwijl er bij mannetjes naar mogelijke verschillen in agressie, reproductief gedrag en uiterlijke kenmerken gekeken werd. Ik heb gevonden dat bij guppies, intra- en interseksuele selectie niet inwerken op dezelfde kenmerken. Vrouwelijke guppies verkozen vooral mannetjes van de interseksuele selectielijn, die in het bezit waren van meer gevarieerde oranje kleurpatronen. Diezelfde mannetjes lieten hun kleuren ook duidelijker zien aan wijfjes. Intraseksueel geselecteerde mannetjes vertoonden dan weer langere staarten (caudale vinnen), die mogelijks hun dominantie status reflecteert.

1.3 Novice abstract

Darwin distinguished two types of selection in his theory of evolution, natural selection and sexual selection. Natural selection chiefly relates to the survival of organisms, whereas sexual selection relates to the organisms' relative success in reproducing. Generally, males compete for females and they can do so in two ways; by being more attractive to females and by competing with other males for females. As such, sexual selection has in the course of evolution, led to so called secondary sex characteristics, which are structures that do not directly benefit the survival of an individual. These characteristics often serve both the purpose of helping to compete with other males for females (armament) as well as serving to attract females (ornament), but these functions do not always overlap. In this study I examine characteristics of the guppy (*Poecilia* reticulata; a tropical fish and model species in sexual selection research) in order to determine which characteristics serve which purpose. This was done by first artificially breeding two types of guppies, which mimic each type of sexual selection, and this was done for three generations of guppies. I then compared the behaviour and characteristics of both males and females between these two types of artificially bred guppies. I found that in guppies, ornaments and armaments are not the same. Males with rarer orange colouration were more attractive to females and they liked to show off their colours, whereas males with larger tails were more dominant. The reason these two types of sexual selection do not overlap in guppies is likely due to the absence of territoriality and resource transfer in the mating systems, which means that females do not gain any direct benefits from choosing dominant males (territory defence and resources).

2 Introduction

> *'And this leads me to say a few words on what I call Sexual Selection. This depends, not on a struggle for existence, but on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring. Sexual selection is, therefore, less rigorous than natural selection.'*
> *~ Charles Robert Darwin, On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life (1859).*

Since Darwin introduced the existence of sexual selection in his *On the Origin of Species* (1859), it has been the subject of much debate. Its basis lies in the observation that some individuals outreproduce others, it therefore differs from natural selection in its sense of success. An individual performing poorly in terms of sexual selection might still reproduce. Indeed, natural selection is considered to supersede sexual selection, and as Darwin himself pointed out, sexual selection is therefore less rigorous than natural selection.

In the above citation[1] it is argued that mainly males outreproduce other males. An important milestone for this was Bateman's principle (1948) some 89 years later. In his study he most importantly pointed out the differential investment of the sexes into their progeny. In most situations, females are the sex that invest most in progeny in terms of energetic expenditure, whereas males are typically the 'cheaper' sex[2]. Bateman's principle is rooted in the anisogamy (dissimilarity of gametes) of sexually reproducing species, with the cheaper mobile sperm and the more expensive sessile eggs leading to discrepancies in reproductive strategy.

Two types of sexual selection are hence distinguished: intrasexual selection and intersexual selection. Intrasexual selection is the competition between or among males over females, which can be considered a limited resource[3]. Male-male competition is a synonym often used. In contrast, intersexual selection is not the competition between females over males, since males are the cheaper sex with 'low-priced' sperm. The manner in which females do attempt to increase their reproductive success, is through direct (e.g. territory and resources) or indirect benefits (e.g. genetic benefits) of choosing the right mate(s). Where the choice of a fit male does not necessarily increase the number of progeny, but rather increases the future survival

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[1] http://darwin-online.org.uk/

[2] Even in sex role reversed species such as seahorses, the Bateman principle still holds, as in that situation males invest more (behaviourally) in progeny, whereas females have the higher reproductive rate.

[3] Note that reproductive success is relative, so a male can skew this both by mating more with females as well as preventing other males from mating with females.

of their own offspring and the reproductive output (indirect) of that offspring. Intersexual selection is therefore generally taken to be a synonym of female mate choice.

Where intrasexual selection derives quite naturally from Bateman's principle, intersexual selection is less self-evident. Female choice deals in 'attractiveness', an ambiguous notion to be selected for to say the least. Fisher (1930) therefore postulated a runaway selection process in which a male trait (conferring an initial adaptive advantage) would become genetically coupled to a female preference for that very same trait. In this manner, males possessing the trait would outreproduce males not possessing said trait, whilst the female advantage gained in possessing the preference lies in producing so called 'sexy sons' or attractive sons herself (the hypothesis is often called the *sexy son hypothesis*). Other hypotheses have been postulated over time, the foremost being: the *handicap model/principle* (Zahavi, 1975), the *compatibility hypothesis* (Yamazaki *et al.*, 1976; dissimilar mating), the *good-genes hypothesis* (Hamilton and Zuk, 1982), and the *sensory bias hypothesis* (Ryan and Rand, 1990). It is important to specify that these hypotheses are not all mutually exclusive and often even overlap to an extent [e.g. the distillation of the handicap principle and the good-genes hypothesis into the *immunocompetence handicap* (Folstad and Karter, 1992)]. In any case, intersexual selection necessitates a trait and a preference for that trait, which are to be genetically coupled.

Thus both forms of sexual selection can lead to dimorphisms between genders, by evolving so-called secondary sexual characteristics or traits. These secondary sexual characteristics arise mostly in males, due to the male reproductive success being the most variable and skewed (Bateman, 1948) in this gender, and thus they are overall subject to stronger sexual selection than females.

On the one hand, where intrasexual selection pressure is highest — e.g. in polygynous mating systems where males can attempt to monopolise group living females and heavily skew reproductive output — dimorphisms tends to take form of increased body size and other traits useful in male-male combat[4] [such as tusks and size in male walruses (*Odobenus rosmarus*; Lindenfors *et al.*, 2002*)*, antlers in stags (Cervidae), etc.].

On the other hand, where intersexual selection pressure is highest and female preference is of most import — e.g. in monogamous, or more polyandry oriented mating systems — more

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[4] This does not imply that male-male combat will always ensue, dominance hierarchies and war of attrition strategies may have evolved to exempt males from costly combat.

colourful and conspicuous elaborations typically evolve to allow for *better* female choice [such as tail length in the long tailed widowbird (*Euplectes progne*; Craig, 1980), or complex song in other songbirds (Passeriformes) and lekking behaviour of displaying males].

It is of course true that male-male competition and female mate choice can act in a complementary fashion and elaborate the same traits (Price and Rodd, 2006) such as in the case of the fathead minnow (*Pimephales promelas;* Hudman and Gotelli, 2007). Intra- and intersexual selection reinforcing one another has been coined the *dual utility model* (Berglund *et al.*, 1996), which is in fact another hypothesis attempting to explain the origin of sexual secondary characters through (mainly) intrasexual selection initiating the trait, and intersexual selection reinforcing the trait further ('armaments are ornaments')[5]. Although the reverse situation is equally plausible. Yet it is not always the case that intra- and intersexual selection select for the same traits, just as in the peafowl[6] (*Pavo cristatos*; Loyau *et al.*, 2005) for instance. This may seem self-evident, since often more than one male trait or character exists. Several hypotheses have been postulated with regard to multiple male traits: the *multiple message hypothesis*, the *redundant signal hypothesis*, the *unreliable signal hypothesis*, *antagonistic co-evolution*, and the *multiple receiver hypothesis* (see Loyau *et al.*, 2005 and references therein).

Guppies (*Poecilia reticulata* Peters 1859), lend themselves exceptionally well for studies on sexual selection. It was first noted by Liley (1966), that sexual selection might operate in this species, and since Endler's study (1980) on natural selection on the guppy, it has become popularised, and even become a model species within the subject. The reason guppies are so suitable, is because they possess a promiscuous mating system (both males and females obtain multiple partners), in which dominance still plays a part (Kodric-Brown, 1992; Bruce and White, 1995). Yet the mating system is without territoriality and resource free (Magurran, 2005).

Territoriality and resources are complicating factors to the study of sexual selection, and intersexual selection specifically. Both holding a territory and holding resources (within that

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[5] In this theory, an ornament can function as a sort of 'badge-of-status', and is used by males in signalling dominance to reduce the potential costs of struggle and combat.

[6] Peafowl is the actual common species name, where it is often mistakenly called a peacock, which is in fact a male peafowl, whereas a peahen is a female peafowl.

territory) provide a more direct merit for females to chose for[7]. Pairing with a male that has greater resources will make it more likely for that female to outreproduce other females with lesser resources, for she can afford to invest more into her progeny. These sort of direct benefits can easily outweigh more indirect genetic benefits *sensu* Fisher, and will often cause female choice and male-male competition to select for the same or similar traits (cfr. *the dual utility model*; Berglund *et al*., 1996). In other words, intrasexual selection will in that case either coincide with female choice, or male dominance may reduce (Reichard *et al.*, 2005) or even override female choice altogether. With both modes of sexual selection present and these complicating factors absent in the guppy system, guppies serve as an ideal organism to study the differential effects of intra- and intersexual selection.

Natural selection is already known to interfere with and restrain sexual selection (e.g.; Endler, 1980), but there is a lack of knowledge on how both modes of sexual selection influence and interact with one another. The aim of this study is to detach the two different types of sexual selection (intra- and intersexual selection) from one another, to find out more on their underlying mechanisms, and better understand the importance of each on the eventual male and female phenotype.

Possible discrepancies are looked for, by maintaining artificial selection lines depicting the two modes of sexual selection in the guppy. Artificial selection consists of a more direct demonstration of selection. It precludes the need of having to quantify reproductive success, since the selected individuals are guaranteed to reproduce. Additionally, the effect of both intra- and intersexual selection are separated, not allowing them to confound one another, or put differently; phenotypes are created in which only one type of sexual selection exerted its effect. This all simplifies comparison, and as such allows for more simplistic experiments without having to statistically partition the effects of intra- and intersexual selection on the male phenotype, as is often done in other experiments and which can be difficult (e.g.: Otronen, 1988; Moore, 1990; Forsgren *et al.*, 1996; Baird *et al.*, 1997; Jones and Hunter, 1999; Reichard *et al.*, 2005; Hudman and Gotelli, 2007; Passos *et al.*, 2013; Řežucha and Reichard, 2014). The apparent disadvantage of artificial selection and laboratory set-ups, are the unnatural conditions. With natural selection being the more rigorous form of selection (Darwin, 1859) and preceding sexual selection, results found under laboratory conditions and

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[7] This assumption does not necessarily hold true for lekking behaviour, where territoriality (*sensu lato*) is present, but a lek is a small area *temporarily* defended from other males to display to females, no direct non-genetic benefits are linked to it.

artificial selection may deviate from the situation in wild populations (with the absence of predators, parasites, environmental fluctuations, etc.).

A point of interest here is of course male mating behaviour, because alternative mating strategies are known to exist in guppies (Reynolds *et al.*, 1993). Male sigmoid displays (courtship) are attempts at consensual mating, whereas gonopodial thrusting (forced copulation) is an attempt to override female consent. These mating strategies are both present in any one guppy male, but they seemingly correspond to inter- and intrasexual selection respectively. As such, it is interesting to investigate whether or not these behavioural strategies alternate with differing modes of sexual selection.

Additionally, multiple male ornamentation is maintained in guppies, generally present in two types of colouration; pigment and structural colouration. The main pigment colourations are orange (carotenoid based) and black (melanin based), whereas iridescence is the structural colouration (Kodric-Brown, 1985). Under the previously mentioned *multiple receiver hypothesis* (see Loyau *et al.*, 2005 and references therein), it is proposed that multiple signalling can be stable due to multiple receivers of the signals, in other words; males and females look at different signals. If this is indeed the situation in guppies, one would expect to find increased male-male signalling in the intrasexual line of selection (signalling dominance, using a *badge-of-status*), and increased male-female signalling in the intersexual line of selection.

Orange (as well as yellow and red) colouration is traditionally considered a trademark for male quality, since carotenoids cannot be produced and have to be taken up from the environment, as well as carotenoids playing a part in immune defence (Kodric-Brown, 1989; Grether, 2000; Grether *et al.*, 2001), making orange colouration the likely more honest signal (Zahavi, 1975) for male quality in intersexual selection (male-female signalling). Black colouration, varies temporal in its expression (Magurran, 2005), there is for instance reduced melanin expression during a simulated predator attack (Price *et al.*, 2008). These changes of colouration are under hormonal (MSH; melanocyte-stimulating hormone) as well as neural control (Price *et al.*, 2008). As such, black colouration can go beyond signalling static properties, assuming honest signalling, such as current quality and motivational state (Price *et al.*, 2008). The role of black colouration is still uncertain.

Furthermore, the female phenotype is explored, mainly with regard to female choice. It may be that females of both lines of selection differentiate in mating behaviour or even mate choice. By controlling reproduction using artificial selection under laboratory conditions, it should theoretically be possible to sway female preference or even recreate a female preference for a trait.

Discriminating between these processes is vital in broadening our understanding of sexual selection, and could help illuminate the potential importance of sexual selection in speciation (Panhuis *et al.,* 2001). As different polymorphs, mating strategies and female preferences may have lead to speciation or might reinforce ongoing speciation.

The effects of artificial sexual selection on male and female phenotypes are thus quantified. Comparing the preference of daughters between the selection lines may provide a better understanding concerning the prerequisites of female preference and its origin. Whereas looking at the male phenotype, will help discern how each mode of selection alters specific male traits. Disentanglement of these phenomena should help clarify theories surrounding sexual selection.

In this study it is hypothesised that: 1) females of each line of selection will have developed a preference for males of their own respective lines, under the assumption that a preference for the artificially selected trait would arise in females, and that this preference would become genetically coupled to the artificial trait; 2) intersexually selected males will perform more display behaviour in order to attract females, whilst intrasexually selected males will try to force more copulations and perform more aggressive behaviours towards females; 3) intrasexually selected males will show more aggression toward their mirror image; 4) and significant differences in male colouration will be found between both selection lines with males of the intersexual lineage showing more orange colouration, and with black colouration possibly being more prominent in males of the intrasexual lineage.

3 Materials and methods

3.1 Species and husbandry

The species used in this study was the guppy (*Poecilia reticulata* Peters 1859), which is a small fish from the Poeciliidae family. Guppies naturally originate from Trinidad, Venezuela, Guyana, Surinam and Tobago; although in recent history they are found more widespread around the world by means of human introduction. Originally this was done in failed attempts at mosquito control, because they feed on insect larvae, and further distribution was likely due to the fact that guppies are now popular aquarium fish. Guppies traditionally occur in small tropical freshwater streams and pools, but may also occur (albeit less abundantly so) in brackish waters, deeper pools and faster streams. (Magurran, 2005)

Guppies are a promiscuous species with both female and male multiple mating. Females are internally fertilised and as such may carry the brood of more than one sire, with the median number of sires being two. The last male to mate is also the most likely to sire the most offspring, but stored sperm can fertilize ova up to eight months. Consequently, sperm competition is present and the male reproductive organ (the gonopodium; the modified anal fin) contains a small hook at its tip, but there is potential for post-copulatory female cryptic choice as well. (Magurran, 2005)

The guppies (*Poecilia reticulata*) used here were descendants of guppies originally caught in 2008 in Trinidad. Since 2012, they have been artificially selected. Only males were used in the artificial selection process, while the females used to breed were taken at random from the stock populations.

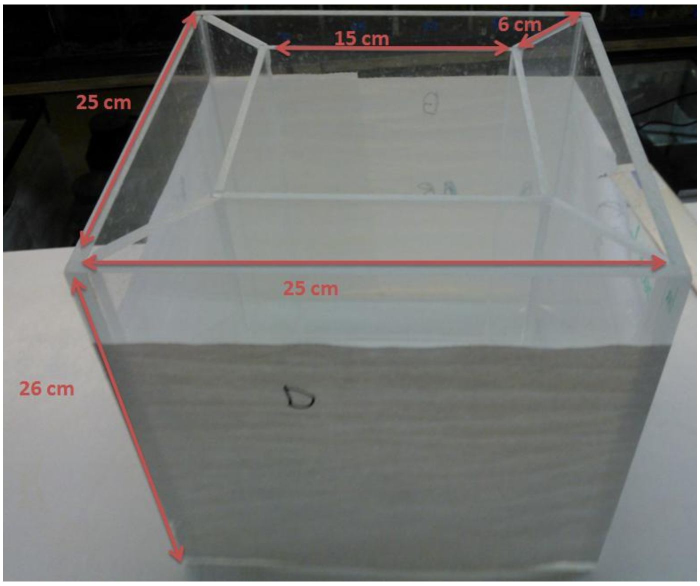

Figure 1: Set-up used to maintain the attractive line of selection. Four males were placed in the four outer compartments, and the focal female was placed in the central compartment.

Three selection lines are maintained. For the first one '**Attractiveness**', intersexual selection is the selective mechanism. Males were selected by placing a female in a central compartment of a plexi-glas container (25 cm x 25 cm x 26 cm; Figure 1), surrounded by four compartments of equal size, in which four focal males were placed. Every minute the preference or non-preference (when the female is at the centre, or at an equal distance of all males) of the female was scored for a total duration of fifty minutes. This was done three times for the same four males using a different female each time. In addition, the bin orientation was shifted 90° for each scoring event, which was done to avoid registering a general preference for a side rather than female preference for a particular male. The proportion of time a male was preferred by the three females was summed for each male. Males of each generation were tested in a tournament set-up, and the five most preferred males were allowed to reproduce, the males of lesser preference were not considered further. Two replicates of selection were used (replicate A and replicate B).

The second line of selection is '**Dominance**', constituting the intrasexual selection mechanism. Males were selected by placing six males in an aquarium (50 cm x 25 cm x 30 cm), each was sketched and recognisable by his colour pattern. A focal male was observed twice for ten minutes, noting the number agonistic behaviours (attacks) initiated and the number of attacks endured by other males. The number of attacks endured was subtracted from the number of attacks initiated to obtain a relative score of dominance within the group of six males. As in the attractive selection line, this process was repeated as many times as required to determine the five most dominant males (used to breed). Similarly, two replicates of selection were created for the dominant line (replicate A and replicate B).

The third line maintained was the '**Random**' line, representing no sexual selection. Random males were picked to mate with females in this line, as such neither female preference nor male dominance influenced mating. These males were not considered in the experiments conducted here.

Selected males each were housed with two virgin females for reproduction, these virgin females were taken from the stock population before reaching sexual maturity. They were housed in see-through plastic boxes (Samla, Ikea) approximating a cuboid in shape and being 35 cm in length, 23 cm in width and 25 cm in height. The water level was kept at around 20 cm and a punctured lid was kept on top to avoid evaporation. The water was continuously filtered, by a small filter being fitted in each box utilising an oxygen pump to stimulate water flow (Hiblow, HP-40).

For all four replicates considered here, five such 'families' (or family units; one male with 2 females) were kept. As soon as young were present in a family unit, the boxes were frequently checked for young males, to be separated from their sisters. Young males can be recognised earliest by the differentiation of the anal fin into the more elongate gonopodium. Discrimination of gender based on the emergence of colour is also possible, but males may already be sexually active by that time. Hence, virgin daughters remained within their family unit, whereas virgin sons were grouped according to their line of selection and their replicate.

These virgin males were kept in a glass cuboid aquaria (50 cm x 25 cm x 30 cm), filtered and kept at 25°C. The aquaria were also partially covered with plastic punctured lids, and similarly water levels were kept at 20 cm high.

Stock fish were kept in 4 large aquaria (100 cm x 40 cm x 40 cm), males and females kept together at random. Likewise these aquaria were filtered continuously and the water level was kept at 23-26 cm in height.

When individual recognition was required, fish were temporarily housed in one of the larger aquaria (100 cm x 40 cm x 40 cm), fitted with 18 compartments. This was done using 16 white flat plastic barriers on the sides of the compartments, and 2 large plastic plates with small holes in them, along the middle. Sand was strewn on the bottom. In-between the two middle plastic plates a thermostat was fitted. The suction for filtration (filter: EHEIM, 2222A) was also located there, with water returning via a PVC pipe fitted over the top back to the compartments (Figure 2). Guppies were only kept separated for the duration of each experiment, after which they were returned to their original aquaria, this because guppies are social fish and extended isolation may cause stress.

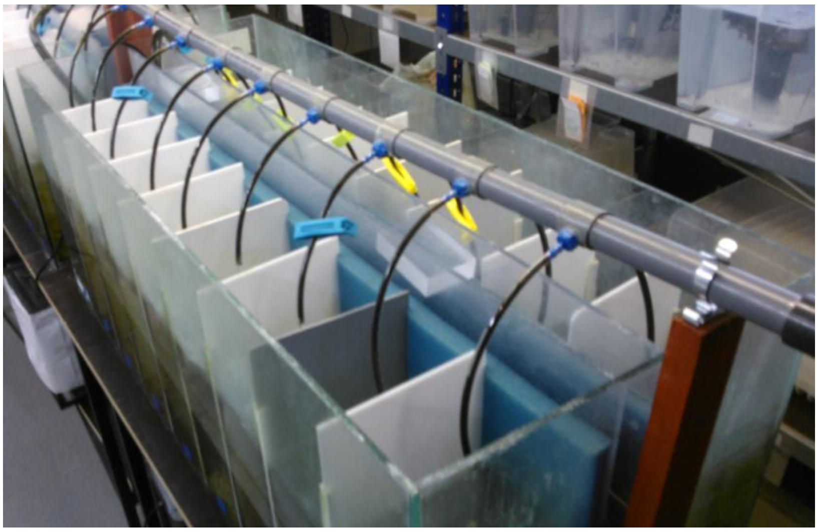

Figure 2: Housing used to allow individual recognition.

All fish were fed at least 2 times a day. Adults were standard fed staple food (Vipagran baby, Sera), whereas young were fed breeding feed (Micron, Sera). All aquaria were stored in the same room, and lights were kept at a day-night regime of 14-12. Water quality was checked (pH, NO_2, NO_3) frequently, and 20% of the water was renewed every week. Water in these aquaria as well as the aquaria used in the experiments were kept at approximately 25°C using thermostats (Aquarium systems Newa; Visitherm; UTX50).

All guppies used in the experiments were of the fourth generation (F_3). And all experiments described below were conducted at a temperature of approximately 24-26°C using thermostats, which were removed at the start of each experiment. Water temperature was tested preceding each trial to see if it was still within the aforementioned range. Long lamps ranging 150 cm in length (TL-D 58W/865, Phillips) overhung the experiments, providing 6500K white light (daylight). All behavioural experiments were conducted between 10:00 and 15:00h, and recorded using a camcorder (JVC, HD Everio, GZ-U515) placed on a stable mount. The scoring of behaviour and time was done using the Jwatcher v1.0 (http://www.jwatcher.ucla.edu) program.

3.2 Female choice

For the female choice experiment, a virgin female daughter of each reproducing family unit was taken, leading to a total of fourteen virgin females ($N_{attr,a}$=4, $N_{attr,b}$=3, $N_{dom,a}$=4 and $N_{dom,b}$=3)[8] to be tested, with seven virgin females belonging to each selection line.

Each of the fourteen virgin females (Houde, 1997), belonging either to a dominant father or an attractive father, underwent three similar choice experiment set-ups. In the first set-up, they chose between a male of an attractive line and a male of a dominant line. In the second set-up, the choice was between two males of an attractive line, and in the third set-up between two males of a dominant line of selection. Due to the fact that all virgin males were housed according to their respective selection line and replicate, there is the possibility that some females in each of these experiments were subjected to half-brothers (same father but different mother) or full brothers (same father and same mother) to chose from.

The experiment was carried out across two weeks (from 9/9/2014 to 19/9/2014). The focal females were temporarily kept separately for individual recognition. To minimise solitary confinement they were randomly divided into two groups, one group for each week of tests.

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[8] $N_{selection\ line,\ replicate}$

Within these groups, the experiments were randomised. Each individual experiment was also assigned a 'trial number', taken together with the randomisation, this allowed for blind scoring of the recordings afterwards.

The experiments took place in two large aquaria (100 cm x 40 cm x 40 cm), consisting of three compartments, divided by two plexi-glass barriers fitted across, each adjoined by a white removable barrier to block preliminary vision from the central compartment. The two side compartments were the smallest (25 cm in length) and held the respective males on each side, the large central compartment (50 cm in length) held the tested female. The central compartments were further visually partitioned into four zones of equal length (12,5 cm; from left to right: Z1, Z2, Z3 and Z4), using red-brown tape on the bottom of the aquaria. (Figure 3)

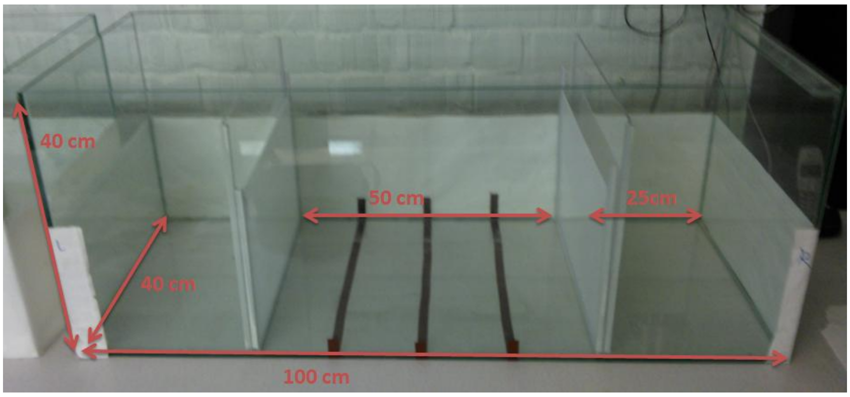

Figure 3: Aquarium used in the female choice and behaviour experiment. Two males were placed in the two outer compartments, and the focal female was placed in the centre. The red tape at the base divide the central compartment into four zones (left to right: Z1, Z2, Z3 and Z4). The solid white barriers were removed after acclimation.

White paper was lengthwise taped around all sides of the aquaria, creating a white background (to increase contrast and visual isolation with the surroundings) reaching just over the water level of around 18-20 cm high. This was not the case on the front, owing to camera instalment in front of the aquaria, directed at the central compartments.

A single experiment then consisted of placing all fish in their respective compartments, noting which male was present where. The fish were allowed to acclimate for five minutes (Cummings and Mollaghan, 2006), then the white barriers were removed and a ten minute recording of the central compartment started. After ten minutes, the white barriers were replaced in their sockets, and the males of the side compartments were switched. The switch was performed to avoid a preference for any one side to be measured. Another minute of acclimation was provided after the switch, after which the barriers were once again removed, and another ten minutes of focal recording took place.[9] Female association time in the 'three-compartment' design is a valid indicator for preference in guppies (Kodric-Brown, 1985; Cummings and Mollaghan, 2006).

Variables measured can be described as either being states (perpetual motion/behaviour) or events (single/countable behaviours). States were measured as times, whereas events were simply tallied. These times and tallies were measured taking into account the respective zones in which the behaviours were performed (read: at which male it was directed; Z1 or Z4).

Such behavioural states were: 1) actively *following* and chasing the male up against the barrier, 2) *hovering* up in front of the barrier facing the male, and 3) all *other* behaviours were simply classified under that very name (all behaviours not denoting preference or proceptivity in any way).

The behavioural events scored were: 1) an *up and down motion* performed by the female (considered to be proceptive) up against the barrier directed at the male (extremely active in her actions of trying to reach the male); 2) an *away and return* motion indicating the male to follow her [as described by Liley (1966)] which could not be done by the male due to the barrier; 3) and a *gliding motion* towards the male (Houde, 1987) which was eventually not strictly observed due to the camera being too far removed from the set-up.

Ultimately, the information concerning behaviours near the males (zone 1 and zone 4) were mainly used (Figure 3), only for the non-receptive behaviours (*other* behaviours) were zones 2 and 3 also relevant.

3.3 Male behaviour

To investigate male sexual behaviour for both lines of selection, each male was placed together with a female from the stock population. This was done in one of the smaller aquaria (50 cm x 25 cm x 30 cm) with all sides apart from the front covered by white paper on the outside (Figure 4). Inside the aquarium some small stones were provided as cover, but no plants were

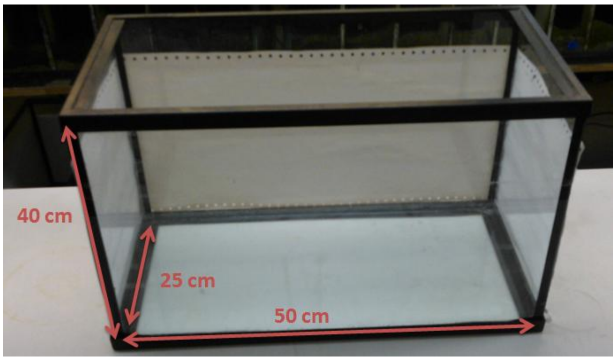

Figure 4: Aquarium used in the male behaviour experiment. The focal male was placed together with one stock female in this open aquarium design.

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[9] No male was used twice in direct succession of a previous experiment. Males used were kept apart, when first a new male was randomly selected from the male housing.

added to optimise visibility. A camera was positioned in front of the aquarium. In total 26 individual males were tested ($N_{attr,a}$=3, $N_{attr,b}$=8, $N_{dom,a}$=6 and $N_{dom,b}$=10)[8]. A single observation lasted ten minutes, preceded by one minute of acclimation time, no more was needed (personal observation; previously tested using stock males and females). Observations were performed from 9/3/2015 to 12/3/2015.

Behaviours were scored similar to Rodd and Price (2006) in an open aquarium design. Male behaviours scored as events were: 1) *biting/nipping* at the female, 2) *nipping* at the female's *gonopore* (possibly assessing female reproductive state and fecundity through pheromones; Herdman *et al.*, 2004), 3) *sigmoid display* and 4) *gonopodial swing* where the male flips his gonopodium (Liley, 1966). The observed states were the following: 1) actively *following* the female, 2) *parallel swimming* and 3) *other behaviours,* meaning no interaction (e.g. foraging). *Parallel swimming* was broadly defined as swimming/hovering beside or in front of the female, trying to stay in view of the female and encircling her. It is important to note that; with it being defined so broadly, this behaviour may also potentially pertain to shoaling behaviour in a non-sexual context.

Observations were performed both in situ as well as using the recordings. The in situ observations benefit from increased visibility and perception of depth, this made it easier to observe the behaviours described as events. The recordings were primarily used to be able to register the proportion of time an individual performed behaviours described as states. The in situ observations were obviously not blinded, but the recordings were blinded (using trial numbers). All observations were performed in random order.

3.4 Mirror aggression test

To explore possible differences in aggression in both lines of selection, a mirror test was used. Guppies have no self-recognition, thus considering they are subjected to an image of themselves, this way of measuring assures body size and potential other factors (e.g. aggression levels of a live opponent) do not interfere with their decision in displaying aggression or other behaviour. Though this method is widely used, validation of this

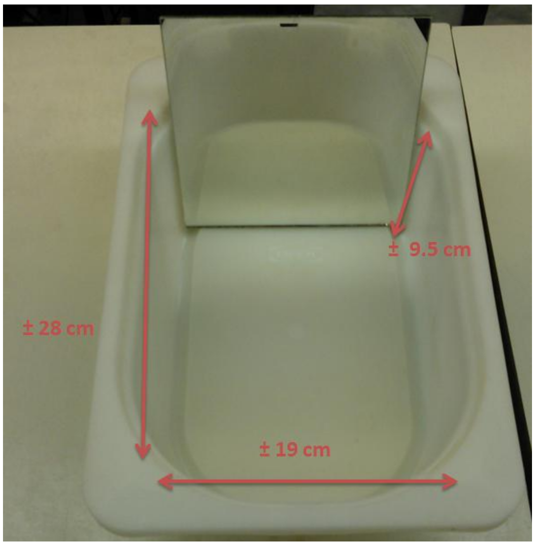

Figure 5: Set-up used for the mirror aggression test. A focal male was allowed to interact with his mirror image for five minutes.

method for individual species is still required, this includes guppies. (Balzarini *et al.*, 2014)

Focal males were put in small white containers (28 cm x 19 cm x 9,5 cm; Figure 5). After one minute for the fish to acclimate, a small mirror was fitted at the back end of each container, after which a five minute recording followed. Water was completely refreshed for each new recording of the next male. Recordings were non-random and unblinded.

An observation started as soon as the guppy approached the mirror. The only event tallied was *biting/nipping* at the mirror image, no sigmoid displays were observed. The timed states were: 1) *swimming parallel* to the mirror, 2) *swimming fast* alongside the mirror (rather aggressive and obvious), 3) *hanging parallel* next to the mirror and 4) *no interaction* with the mirror image (previous experiments Huyghe K., unpublished).

A total of 60 individuals ($N_{attr,a}$=8, $N_{attr,b}$=16, $N_{dom,a}$= 16 and $N_{dom,b}$=29)[8] were recorded and observations were carried out from 22/10/2014 - 24/10/2014.

3.5 Male morphology

Male colouration was quantified using photographs taken with a stereoloupe with built in camera (LEICA, TL5000). In order to do this, guppies were sedated by being exposed to water with solved clove oil, put on a dry petridish and dried off (any excess water increased reflection), and were subsequently photographed on both sides. After no longer than two minutes, fish were released in clean water to recover (all recovered well within ten minutes). Each of the 60 individuals ($N_{attr,a}$=8, $N_{attr,b}$=16, $N_{dom,a}$= 16 and $N_{dom,b}$=29)[8] were photographed under the exact same optical conditions (light and zoom), and this was done over two consecutive days (23/10/2014 - 24/10/2014).

Photographs were then scaled (millimetres) and measured using the ImageJ v1.47 software (http://imagej.nih.gov/ij/). For both the left and right side of each male guppy, the following was measured: body length; tail (caudal fin) length (caudal fin length); total area (i.e. tail and body combined); and the amount of black, orange and iridescent spots, and their respective surfaces.

From these measurements, proportions were calculated for each fish. The left and right side photographs were first taken together (summed) for each area of colour, as well as for total body area. Dividing the total area of any one colour by the respective total area of the fish then yielded proportions of colouration. This was done to correct for differences in sizes between these individual males, so as to solely compare relevant colouration. A larger fish will, logically, on average show more colouration (see also Appendix B for correlations), as well as studies having found that total body size also affects mate choice in guppies (Reynolds and Gross, 1992; Karino and Matsunaga, 2002), this appears to be a general trend in fish for both males and females (Magurran, 2005; and references therein). Since it can probably be considered the oldest and most honest form of fitness signalling. Relative differences and hence proportions, were considered the most relevant for area of colouration.

Subsequently, body size was regarded separately. Body length, tail length, body area and tail area all heavily intercorrelate (Appendix A), making them good predictors of one another. Only body length and tail length were considered here and used as a proxy for body size, since both tail area and body area showed higher measurement errors (Sokal and Rohlf, 1981[10]; Table 1), and would decrease statistical power. For body length and tail length, the mean of the left and right side photographs were taken for each individual.

The considerably higher measurement error found for tail area (27.5%) can be attributed to the spread of the tail. For the photographs, the males' tails were spread out to their maximum extent by the observer, while trying to minimise damage to the tail. Success in this endeavour varied considerably (both spread- and damage-wise), hence the high measurement error.

Table 1: Measurement errors on male morphometry following Sokal and Rohlf (1981)[10].

Measurement error	
Body length (mm)	3.3 %
Body area (mm²)	4.5 %
Tail length (mm)	9.0 %
Tail area (mm²)	27.5 %

3.6 Statistical analysis

All data were organised and transformed using Microsoft Excel (2007), and all statistical analysis were carried out using the R 3.0.1 program (http://www.R-project.org/). Generalised linear models were constructed separately for each dependent variable.

The dependent variables used were either tallies (e.g. the number of black coloured spots counted, the number of times a male nipped at the female, etc.) or measurements converted to

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[10] $\%ME = \frac{s^2_{within}}{s^2_{within}+s^2_{among}} * 100$

$[with\ s^2_{within} = MS_{within}\ and\ s^2_{among} = \frac{MS_{among}-MS_{within}}{m}\ (with\ m = \#\ of\ repeated\ measurements)]$

proportions (e.g. orange area relative to the total area, the proportion of the total time spent following a female, etc.). Consequently, count data were square root transformed ($\sqrt[2]{x}$), and proportions of totals were arcsine transformed ($\sin^{-1}(\sqrt[2]{x})$) to reach normality.

The independent variables used in each model always contained the male line of selection (attractive or dominant), as well as their replicates (A or B). The first experiment (3.2 Female choice) consisted of three experimental set-ups which were analysed together, necessitating the addition of several terms. These were the following: the origin of the female (daughter of an attractive or dominant father), the set-up (1, 2 or 3), cover (present or absent)[11], recording (first or second recording; original or reversed situation) and the identity of the focal female. Also, the only two possible and meaningful interactions were tested: the interaction between the origin of the male and the origin of the female (to see for example if daughters of dominant fathers preferred sons of the same type of father), and the interaction of the origin of the female and the experimental set-up (to search for differences in behaviour when surrounded by different type males).

The assumptions of normality and homoscedasticity of the final model were graphically explored, and ANOVA analysis (F test) was subsequently used[12] on each model. Significance levels for all tests were set at $\alpha=0.05$, and all probabilities reported are two-tailed unless stated otherwise.

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[11] Some cover was provided in the very centre of the central compartment consisting of some small stones and a plastic aquarium plant. But this only after the 21st trial, to reduce stress in the tested females.

[12] ANOVA is nonetheless robust when it comes to small deviances from normality and homoscedasticity.

4 Results

4.1 Female choice

Overall, females were found more in zones nearest to attractive males, than in zones near dominant males ($F_{1,83}=5.108$, $p=0.025$).

Females actively *followed* attractive males significantly more than they did dominant males ($F_{1,83}=8.022$, $p=0.005$), this regardless of whether it was a daughter of an attractive or dominant father ($F_{1,41}=1.200$, $p=0.277$; Figure 6).

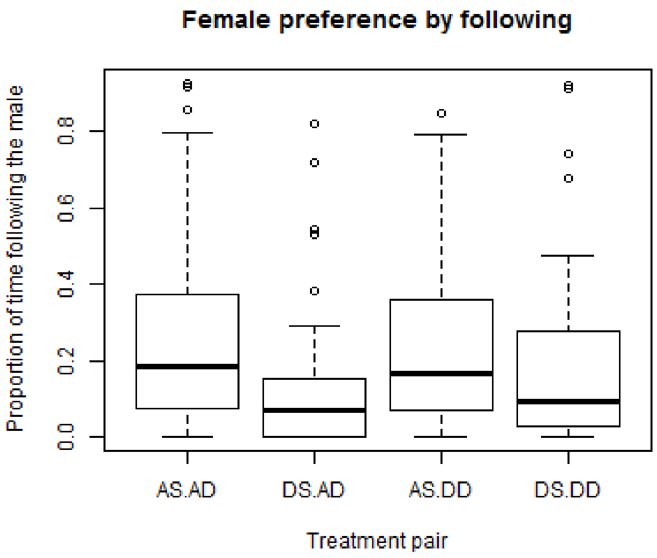

Figure 6: The proportion of time females followed respective males during the ten minute recordings. With AS = attractive son, DS = dominant son, AD = attractive daughter and DD = dominant daughter.

No preference was found for *hovering* near males ($F_{1,83}=1.786$, $p=0.184$), only individual differences among females were present ($F_{10,11}=2.240$, $p=0.018$). This behaviour was found very inconsistently and seldom, making it a bad predictor of preference.

Behaviours jointly classified as *other* behaviours (or in other words: non-proceptive behaviours), were found to only significantly differ between females ($F_{10,11}=5.515$, $p<0.001$), showing differences in proceptivity between females. An indicative yet insignificant difference was found in the aspect of cover ($F_{1,63}=2.884$, $p=0.092$), more non-proceptive behaviour was found in the absence of cover, indicating that stress may indeed have been a factor and that the addition of cover ultimately proved useful.

The very active *up and down motion* was found to be directed significantly more towards attractive males ($F_{1,83}=4.913$, $p=0.028$). A significant difference was also found between females ($F_{10,11}=2.989$, $p=0.002$), again marking individual differences in either proceptivity or perhaps quite simply activity. An interaction effect was found between the origin of the daughter and the experimental set-up ($F_{2,27}=3.660$, $p=0.028$). In the third set-up (with a female being flanked by two dominant males), daughters of attractive fathers showed more *up and down movements* than did daughters of dominant fathers. Overall, this behaviour was performed the most in the second set-up, where a female was flanked on both sides by attractive males.

The *away and return motion* as previously described, was performed significantly more in the second set-up (female flanked by two attractive males; $F_{2,55}$=6.011, p=0.003). No significant effect of males (attractive or dominant) was found in this case ($F_{1,83}$=0.168, p=0.682). The zigzag motion violated the ANOVA assumptions of normality, homoscedasticity and non-linearity; and since no non-parametric alternative exists for the ANOVA-model, this was not investigated further[13].

Overall, attractive males were preferred by daughters of both attractive and dominant fathers, hence daughters of dominant fathers did not show any inclination of preferring dominant males (Figure 6).

This is also confirmed when regarding the results of the first experimental set-up separately (where females were given the choice between a dominant male and an attractive male), again females were found to spend the majority of their time *following* the attractive male ($F_{1,27}$=8.066, p=0.007), regardless of their own origin ($F_{1,13}$=3.222, p=0.081; Figure 7).

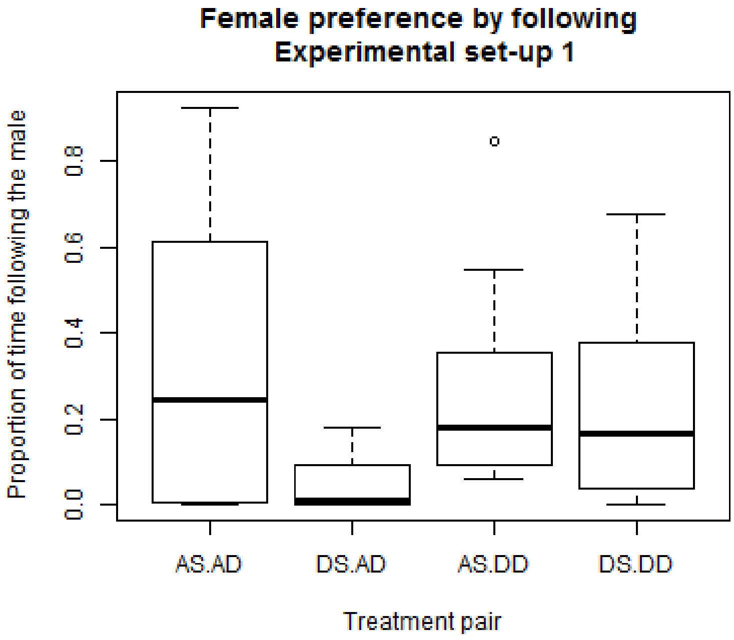

Figure 7: The proportion of time females followed respective males during the ten minute recordings, in experimental set-up 1. With AS = attractive son, DS = dominant son, AD = attractive daughter and DD = dominant daughter.

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[13] Executing multiple individual non-parametric tests, which incidentally already suffer from a lower statistical power, would additionally lead to an increased possibility of type I errors (false positives).

4.2 Male behaviour

During the entire experiment, no copulations were observed, neither consensual nor sneaky/forced (gonopodial thrust).

It was found that attractive males performed significantly more *gonopodial swings* than dominant males ($F_{1,9}$=10.692, p=0.003; Figure 8 A; Table 2) as well as them *swimming* a significantly larger proportion of the time *parallel* to the female than did dominant males ($F_{1,9}$=7.005, p=0.015; Figure 8 B; Table 2). However, attractive males did not perform more *sigmoid displays* than did dominant males ($F_{1,9}$=1.030, p=0,321; Table 2).

Additionally, dominant males were not found to *nip* more at the female *gonopore* ($F_{1,9}$=0.053, p=0.820; Table 2), nor more at the their bodies ($F_{1,9}$=1.623, p=0.216; Table 2), nor more general *nipping/biting* overall ($F_{1,9}$=0.357, p=0.556; Table 2). Dominant males did not even spend more time *following* or actively chasing females ($F_{1,9}$=0.717, p=0.406; Table 2).

Table 2: The ANOVA-model results for male behaviours displayed in the presence of a random stock female. 'Line of selection' indicates a possible difference between males selected for attractiveness or dominance. 'Replicate' indicates possible differences between the replicates of the lines of selection (four replicates; attractive A and B, and dominant A and B). Df= degrees of freedom (between and within). Significance levels were taken to be: p<0.001=*; p<0.01=**; p<0.05=* and p≤0.1=● (meaning indicative rather than significant). 'a' denotes square root transformed data (events/counts), 'b' denotes arcsine transformed data (states).**

Male behaviour					
Behaviour	**Independent variable**	**Df**	**F-value**	**P-value**	**Significance level**
Sigmoid displays^a	Line of selection	1,9	1.030	0.321	
	Replicate	2,2	0.435	0.653	
Gonopodial swings^a	Line of selection	1,9	10.692	0.003	**
	Replicate	2,2	7.141	0.004	**
Nip at female body^a	Line of selection	1,9	1.623	0.216	
	Replicate	2,2	1.192	0.322	
Nip at female gonopore^a	Line of selection	1,9	0.053	0.820	
	Replicate	2,2	2.239	0.130	
Total nips at female^a	Line of selection	1,9	0.357	0.556	
	Replicate	2,2	1.819	0.186	
Following^b	Line of selection	1,9	0.717	0.406	
	Replicate	2,2	1.458	0.254	
Parallel swimming^b	Line of selection	1,9	7.005	0.015	*
	Replicate	2,2	6.108	0.008	**
Other^b	Line of selection	1,9	1.180	0.289	
	Replicate	2,2	6.185	0.007	**

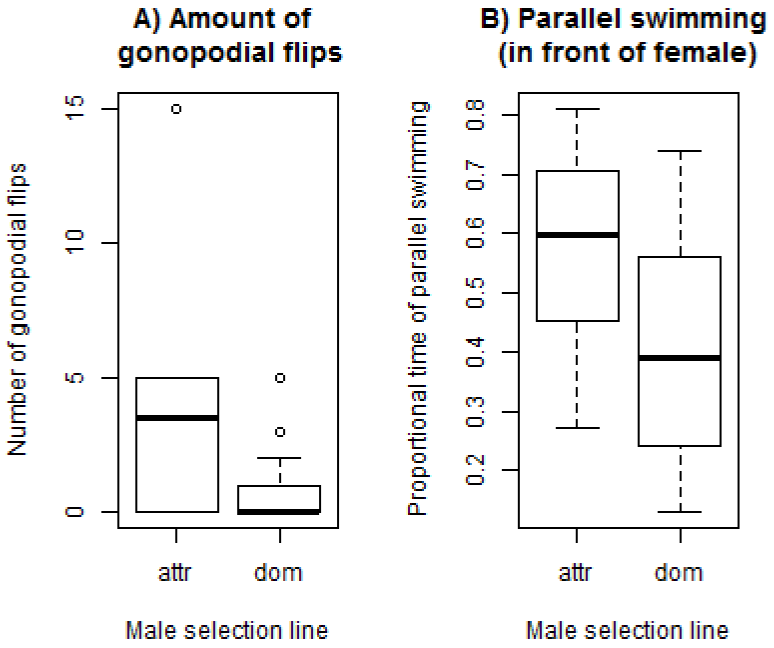

Figure 8: A) The difference in the amount of gonopodial swings performed at a female by attractive males (attr) and dominant males (dom). B) The difference in the proportion of time an attractive male (attr) and a dominant male (dom) spent swimming parallel (or in front) of a random female.

4.3 Mirror aggression test

Overall, no significant differences could be found between both lines of selection for the mirror aggression test. The only significant differences were found between replicates, where in the attractive line of selection, replicate A differed from replicate B in all behaviours apart from *parallel swimming*. Individuals from replicate A showed a significantly lower amount of bites ($F_{2,7}$=6.626, p=0.002; Table 3), spent less of their time *swimming fast* next to the mirror ($F_{2,7}$=5.152, p=0.008; Table 3), *hung* more in *parallel* to their mirror image (passive behaviour; $F_{2,7}$=5.714, p=0.005; Table 3), and interacted less with their mirror image ($F_{2,7}$=3.778, p=0.028; Table 3), than did individuals from replicate B.

Table 3: The ANOVA-model results for male behaviours displayed in the presence of a mirror image. 'Line of selection' indicates a possible difference between males selected for attractiveness or dominance. 'Replicate' indicates possible differences between the replicates of the lines of selection (four replicates; attractive A and B, and dominant A and B). Df= degrees of freedom (between and within). Significance levels were taken to be: $p<0.001$=*; $p<0.01$=**; $p<0.05$=* and $p\leq0.1$=● (meaning indicative rather than significant). 'a' denotes square root transformed data (events/counts), 'b' denotes arcsine transformed data (states).**

Mirror aggression test					
Behaviour	**Independent variable**	**Df**	**F-value**	**P-value**	**Significance level**
Biting/nipping[a]	Line of selection	1,23	0.079	0.779	
	Replicate	2,7	6.626	0.002	**
Fast swimming[b]	Line of selection	1,23	1.959	0.166	
	Replicate	2,7	5.152	0.008	**
Parallel swimming[b]	Line of selection	1,23	1.992	0.163	
	Replicate	2,7	1.616	0.206	
Parallel hanging[b]	Line of selection	1,23	0.320	0.573	
	Replicate	2,7	5.714	0.005	**
No interaction[b]	Line of selection	1,23	0.174	0.678	
	Replicate	2,7	3.778	0.028	**

4.4 Male morphology

A significant difference was found in the proportionate amount of orange area different type males have ($F_{1,23}$=8.285, p=0.005; Table 4; Figure 9) as well as in their number of orange spots ($F_{1,23}$=11.834, p=0.001; Table 4; Figure 9). As can be seen in Figure 9 (untransformed data), dominant males showed more orange area and spots than did attractive males. However, the difference is not very large since both boxplots still overlap considerably, and the main point of interest is in fact the higher variance of orange in attractive males, as is also clear from Figure 9. A significant difference was also found in the amount of iridescent spots ($F_{1,23}$=6.708, p=0.012; Table 4), with dominant males on average having more of such spots.

Tail length was found to be significantly different between the two lines ($F_{1,23}$=4.8648, p=0.031;Table 4), and was on average 0.188 mm longer in dominant individuals. However, such a difference was not found with regard to body length ($F_{1,23}$=0.174, p=0.678; Table 4) despite the high intercorrelation between the tail length and body length (Appendix A). The increased tail length did not translate into a significantly larger total length for dominant males ($F_{1,23}$=0.118, p=0.732; Table 4). As is clear from Figure 10, the difference was only in intercept and not in coefficient, the descendants of the dominant lineage had only slightly longer tails. Significant differences were also found between replicates for tail length and body length ($F_{2,7}$=26.542, p<0.001 and $F_{2,7}$=26.736, p<0.001 respectively; Table 4), with a longer tail length being in favour for each of the A replicates in contrast to their B replicate counterparts.

Table 4: ANOVA-model results for male colouration and morphometry. 'Line of selection' indicates a possible difference between males selected for attractiveness or dominance. 'Replicate' indicates possible differences between the replicates of the lines of selection (four replicates; attractive A and B, and dominant A and B). Df= degrees of freedom (between and within). Significance levels were taken to be: p<0.001=*; p<0.01=**; p<0.05=* and p≤0.1=● (meaning indicative rather than significant). 'a' denotes square root transformed data (counts/events), 'b' denotes arcsine transformed data (proportions of total area of the guppy that were coloured). Data on morphometry 'c' were untransformed and in mm.**

Male colouration						
Measurement	**Colour**	**Independent variable**	**Df**	**F-value**	**P-value**	**Significance level**
Number of spots^{a}	Orange	Line of selection	1,23	11.834	0.001	**
		Replicate	2,7	0.155	0.856	
	Black	Line of selection	1,23	2.561	0.114	
		Replicate	2,7	1.667	0.197	
	Iridescence	Line of selection	1,23	6.708	0.012	*
		Replicate	2,7	2.545	0.086	●
Proportion coloured^{b}	Orange	Line of selection	1,23	8.285	0.005	**
		Replicate	2,7	0.230	0.795	
	Black	Line of selection	1,23	0.254	0.616	
		Replicate	2,7	1.409	0.252	
	Iridescence	Line of selection	1,23	3.658	0.060	●
		Replicate	2,7	1.030	0.363	
Morphometry^{c}	Tail length	Line of selection	1,23	4.865	0.031	*
		Replicate	2,7	26.542	< 0.001	***
	Body length	Line of selection	1,23	0.174	0.678	
		Replicate	2,7	26.736	< 0.001	***
	Total length	Line of selection	1,23	0.118	0.732	
		Replicate	2,7	29.457	< 0.001	***

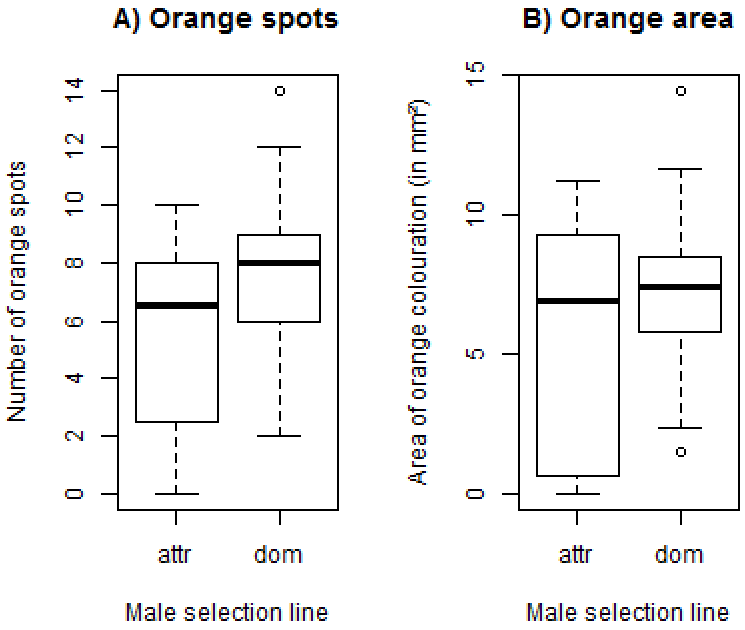

Figure 9: A) The difference in the number of orange spots in both types of males, with attr = attractive males and dom = dominant males. B) The difference in area of orange colouration in both types of males, , with attr = attractive males and dom = dominant males.

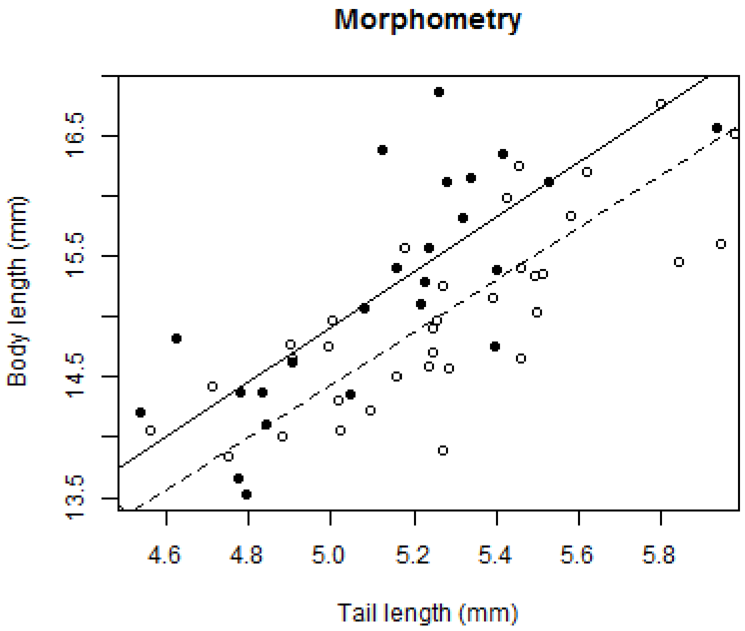

Figure 10: Differences in morphology. Tail length (in mm) plotted against body length (in mm). Black points represent males of the attractive lineage with the full line denoting their linear regression, whereas open points are males of the dominant lineage with the dashed line denoting their linear regression. Males of the dominant lineage had significantly larger tails, as denoted by the higher x-intercept.

5 Discussion

5.1 Female choice

Overall, males of the attractive line of selection were preferred by both daughters of the dominant line and the attractive line, which was mainly done by following them and preferring their presence. This simply due to the fact that, well, attractive males are attractive. Daughters of the intrasexual line did not prefer dominant males. The most obvious reason for dominant daughters not preferring dominant sons is of course the small number of generations that have passed in the selection process. Artificial selection after three generations may yield some effects, but a shift in preference would take a significantly longer time still.

Where intersexual selection undeniably necessitates the presence of both a male trait and a female preference for that same trait, no such requirement is strictly needed where dominance and male armaments for male-male competition are concerned. This follows from the benefits of mating with more dominant males, which are usually of a more direct nature (e.g. territory, increased predatory protection and resources) for females. Guppies show little to no territoriality and they have a polygynandrous (or promiscuous) mating system (Magurran, 2005), making those direct benefits of preferring a dominant male smaller or even non-existent. Yet despite these considerations, there are reasonable arguments to assume that daughters of dominant fathers, would possess a preference for dominant males if artificial selection is to continue. Through the process of artificial selection used, a female mating with a dominant male would have dominant sons. Of those sons, the most dominant of these, would again be guaranteed to mate with two virgin females and would be the only sire of those broods. In this manner, the process of artificial selection mimics the indirect benefits of the *sexy son hypothesis* in the dominant line just as it does in the attractive line. The increased reproductive success of the dominant sons, assured through artificial selection, can provide the indirect benefit to the mother.

The only behaviour where daughters of different lineages differed, was the up and down motion, when they were flanked by two dominant males. In situations where females were flanked by two dominant males (set-up 3), attractive daughters showed more up and down movements than did dominant daughters, but overall this behaviour was performed most when a female was flanked by two attractive males (set-up 2). The up and down motion was clearly directed at the male on the other side of the plexi-glass, and could be construed as an attempt of the female to reach the male compartment. As such, differences in the up and down motion imply differences in female sexual motivation or proceptivity. The highest sexual motivation thus occurred when surrounded by attractive males, regardless of female origin. Attractive daughters did not show an equal reduction in sexual motivation as dominant daughters, when flanked by two dominant males. These females might have a higher overall propensity to mate. At first sight this might seem contrary to expectation, because intersexual selection by definition favours female choosiness, but increased female reproductive motivation does not necessarily imply a decrease in mate discrimination, nor does it imply an increase in promiscuity.

Naturally, individual differences were found in-between females, this for the up and down motion as well as for the behaviours commonly denoted as 'other'. Females thus differ in either their activity levels or their propensity to mate. Whichever the case, it illustrates the importance of conducting dichotomous choice trials for female preference. As Magurran (2005) also pointed out, sometimes no-choice trials are performed to assess female choice, while in other studies choice trials are used. In no-choice trials; a 'one male and one female' set-up is utilised, and all females' behaviour is subsequently used to quantify overall mate choice. This is done to maintain an open aquarium design, for the sake of allowing a female access to all possible cues (visual, olfactory and behavioural). But consequently, since females differ individually in their promiscuity, their behaviour can not be simply compared to other females. This then requires multiple testing of each female with different males, so that observations within females can be compared. This leads to the complication that earlier experience affects female mate choice (Breden *et al.*, 1995; Rosenqvist and Houde, 1997). With this in mind, I argue that dichotomous mate choice trials should always be opted for, particularly when investigating ornamentation and female preference.

Overall, attractive males were preferred by females, indicating a discrepancy in guppies between intra- and intersexual selection. The two modes of selection do not coincide in guppies, for if that were the case, no difference in preference should have been found. This is contrary to the situation in many other species, such as: the horned beetle (*Coprophanaeus ensifer*; Otronen, 1988), the sand goby (*Pomatoschistus minutus*; Forsgren *et al.,* 1996), the fathead minnow (*Pimephales promelas*; Hudman and Gotelli, 2007), the crested auklet (*Aethia cristatella*; Jones and Hunter, 1999) and the annual killifish (*Austrolebias charrua*; Passos *et al.*, 2013); where intra- and intersexual selection do coincide. In all of the above species, intra- and intersexual selection acted in concert on body size, apart from the crested auklet, where the crest ornament served as an attractive trait as well as a *badge-of-status*.

In concurrence with the results for guppies here, intra- and intersexual selection do not coincide in peafowl (*Pavo cristatus*; Loyau *et al*., 2005) and in the pond dragonfly (*Libellula luctuosa*; Moore, 1990), and select for different traits. The main difference between all of these results can likely be attributed to the fact that in guppies, peafowl and pond dragonflies; no real territoriality, no paternal investment and no resource transfer exists. Peacocks and male pond dragonflies do show temporary territoriality. Peacocks compete for display leks, with older and more dominant males controlling central leks, whereas male pond dragonflies compete for shore locations where females might oviposit. Both these forms of temporary territoriality provide no real further benefits to females.

On a different note, with regard to female mate choice and reproductive success, I would like to point out the necessity of investigating female reproductive success. Male reproductive success varies more than does female reproductive success (Bateman, 1948), this simplifies measuring differential reproductive success in males. However, female reproductive success through mate choice is also highly relevant to Fisher's (1930) hypothesis, yet data on female reproductive success is limited. None of the above studies (Otronen, 1988; Moore, 1990; Forsgren *et al.*, 1996; Jones and Hunter, 1999; Loyau *et al*., 2005; Hudman and Gotelli, 2007; Passos *et al.*, 2013) on sexual selection considered female reproductive success. Even if male reproductive success is considered, the further reproductive success of each male's offspring should also be taken into account. More long term studies are required to increase our knowledge on female reproductive success, female mate choice and potential sexual conflicts therein.

5.2 Male behaviour

Several studies found an association between display rate and female attractedness (Bischoff *et al*., 1985; Farr, 1980). If display rate and attractiveness are indeed correlated, then it is expected that males chosen for attractiveness should display more. Males from the attractive lineage did not show any more displays than did individuals of the dominant lineage. It should be noted that only the number of displays (in other words the frequency of courtship display) was considered here. Sigmoid display length and intensity were not quantified and regarded. As Houde (1997) also argues, there might be a difference in quality of displays, rather than a difference in quantity.

Nevertheless, males from the attractive selection line were found to have swum more frequently in parallel to the female. Seeing as the behavioural state was defined as: '*swimming* *or hovering beside or in front of the female, trying to stay in view of the female and encircling her*'; it is easily interpreted as the more obvious strategy for the attractive lineage of males to apply. If this behaviour (as previously suggested; 3.3 Male behaviour) pertains to shoaling behaviour and is non-sexual, no difference in between the two lines of selection should have been found.

Attractive males also performed more so called gonopodial swings. Liley (1966) viewed these gonopodial swings as orientation and non-courtship behaviour, and pointed out that is superimposed on multiple behaviours. However, Liley did also consider: '*Another possibility is that Gonopodial Swinging prepares the gonopodium and related structures for Copulation. However, as males appear to be capable of Copulating almost immediately in an encounter with a female without a number of preliminary Gonopodial Swings, it seems unlikely that the latter explanation applies*'. Despite Liley's reluctance, this alternate explanation may not have been far from the mark. Řežucha and Reichard (2014; investigating the closely related *Poecilia wingei* or Endler's guppy) interpreted Liley's alternate possibility as the male loading the gonopodium with sperm. Because they found a positive association of sigmoid displays (courtship) with gonopodial swings, and taken together with Pilastro and Bisazza (1999) findings that courtship mating on average transfers more sperm than does sneaky mating, Liley's explanation does become likely. The results acquired here, of attractive males (representing a courtship strategy) showing more gonopodial swings, agree with this interpretation. Although, I would not exclude the possibility that it might nonetheless have a function in sexual signalling, either visually or olfactory (e.g. pheromones).

Males from the dominant lineage did not significantly perform anymore gonopore nips or regular bites/nips at females. It has been suggested by Herdman *et al.* (2004) that males nipping at the female gonopore is a male's way of assessing the female reproductive state, potentially through pheromones. This suggestion was based off of a study by Crow and Liley (1979), where they found that males were attracted to water that previously held females, but not to water that previously held ovariectomised females. Taken together with the fact that the fused ovaries lead directly to the gonopore through a short gonoduct (Magurran, 2005), this is a very reasonable suggestion. From personal observation, however, these gonopore nips seemed to scare females. If this behaviour is indeed part of the 'dominant mating strategy' (forced non-consensual mating), one would have expected to find an increase of this behaviour in the dominant lineage. The male behaviour experiment conducted here, therefore does not disprove Herdman *et al.*'s (2004) suggestion, but neither can it confirm it.

Furthermore it should be pointed out that, both strategies, meaning sneaky matings and consensual mating with courtship (sigmoid) displays, are applied by all males in the wild as well as in laboratory studies. The more intersexual oriented strategy (courtship and display) and the more intrasexual oriented strategy (dominance and forceful copulation) do not exclude each other, and the mating behavioural strategies should be seen as continuous rather than discrete. Their use appears to vary mostly in relation to their: 1) social environment, with sneaky (forced) matings occurring more frequently in scenarios with increased male-male competition; 2) and their ecological environment, with display behaviour occurring less frequently and sneaky mating occurring more frequently under higher predation pressure for instance (Magurran, 2005). On average, sigmoid displays were performed the most throughout this experiment, this in the absence of predation and male competition. There is, however, indirect evidence (Řežucha and Reichard, 2014; who found a negative correlation between the number of sigmoid displays and gonopodial thrusts in naïve males) as well as direct evidence (Evans, 2010) for a genetic basis to male sexual behaviour, and its covariance to other male traits such as sperm motility (constituting the trade-off in competition between both strategies). The results here seemingly agree with these studies, as differences in reproductive behaviour were found between the selected lines.

5.3 Mirror aggression test

Dominant males did not show more aggression towards their mirror image than did attractive males. This finding is contrary to expectation, and may have to do with methodology. Balzarini *et al.* (2014) argues that despite the numerous advantages mirror tests have to offer, they may vary in applicability across species, and first need to be validated. In their study they found that the mirror aggression test was consistent with live opponents in only one of three fish species tested. The social structure of these may be at the basis (with the accuracy of the test increasing with the group living species), as well as the behavioural repertoires: '*In interactions between live fish, often lateral displays are shown in an antiparallel way (the two fish heading in opposite directions, head to tail. This pattern is disrupted by the mirror image, which is head-to-head to the test subject, creating a potentially confusing signal*'. The interactions with the mirror in this experiment were also predominantly lateral (personal observation) as opposed to frontal (frontal displays would not be distorted).

In spite of this argument, differences in aggression were still found, but in between replicates rather than between selection lines. Indicating that the method must have relevance, since all behaviours apart from parallel swimming were highly significant. This can barely be called a statistical artefact. It is therefore very likely that there simply are no differences in aggression between the two lines of selection.

A possible explanation pertaining to the differences found in replicates may be found in social rearing environment. Males were separated from females before reaching sexual maturity and housed in an exclusive male environment before the experiments took place. It has been shown, for instance, that social (rearing) environment and sex ratio affect male behaviour (Jirotkul, 1999), which may have likewise confounded the results. Since males were housed according to their replicates, the differences in replicates support this view. As in both the attractive lineage ($N_{attr,a}$=8 versus $N_{attr,b}$=16) as well as in the dominant lineage ($N_{dom,a}$= 16 versus $N_{dom,b}$=29), replicates A were housed with fewer males than their B replicate counterparts. This may have caused the consistently lower rates of aggression in the A replicates. Strictly speaking, this is no change in sex ratio, since no females were present in all male housings, but more males still resulted in higher levels of aggression.

5.4 Male morphology

Dominant males had a significantly larger proportion of their total body covered in orange, but however significant this effect was, the actual difference was very small (Figure 9). Most studies find the exact opposite to be true; that is that orange colouration (both chroma and area of colouration) are preferred by females (e.g.: Kodric-Brown, 1985; Brooks and Endler, 2001), as opposed to this colouration playing a part in intrasexual competition.

The thing that stands out in this study, is the difference in variance for the area of orange colouration as well as for the number of spots (see Figure 9; the number of spots may be a proxy to the complexity of colouration patterns; Brooks and Endler, 2001) that attractive males had, in comparison to dominant males. This may be a simple artefact of sample size, as the sample size was considerably lower for the attractive males (N_{attr}=24 versus N_{dom}=45). Alternately, if it is assumed to not be a statistical artefact, this observation agrees with several studies done with regard to negative frequency dependent sexual selection in guppies (Farr, 1977; Hughes *et al*., 1999; Eakley and Houde, 2004). According to these studies as Farr (1977) puts it: '*A female who chooses a rare or different male will produce offspring exhibiting more genetic heterozygosity, since there is a lower probability that she shares, many of her genes with a rare male than with a common male. By choosing rare males, a female not only insures a greater heterozygosity in her offspring, but contributes to* *maintaining a higher level of polymorphism in the population*[14].' Farr, further argues that heterogeneous environments could maintain such a type of selection, and that tropical seasonality in guppy habitat results in such selection, by washing out populations during the rainy season which are subsequently re-established by few females and males. It would therefore be advantageous for females to mate with novel and different type males, to counter-act inbreeding depression.

A further consideration is of course: what is indeed important, quantity (orange area) or quality (chroma)? Magurran (2005; with references therein) states that: '*the size and distribution of orange spots is under genetic control [...], whereas the chroma (colour saturation) is affected by diet [...] Most studies that attribute a mating advantage to carotenoid colours measure area, rather than chroma.*' Chroma were not measured here, only area, and with that being the case; attractive males might still differ in chroma, even under standardised laboratory conditions (where the diet is the same for all males), if attractive males invest relatively more of their carotenoids into ornamentation than do dominant males.

No effect for black colouration was found. Yet black spots or stripes became distinctly more apparent when a female was introduced to male in the aquarium (personal observation), and this happened in all males. Therefore it is hard to fathom that black colouration does not play a part in reproductive context. It is perhaps relevant to both strategies investigated here, in equal measure, and as such may serve a role as both a *badge-of-status* as well as an ornament.

It was also found that dominant males had significantly more iridescent spots than attractive males had. However, the same result was not found when it came to iridescent area as a proportion of total body area. This might be taken to mean that the iridescent spots of attractive males were whole, while those of dominant males were more frequently subdivided and complex (differences in iridescence area did not reach the significance level by a small margin; p=0.060; Table 4). I personally found it hardest to measure iridescence, because iridescence is a structural colouration, and its measurement depends largely on the angle formed between the light reaching the object of study and the point from which it is observed. Even though constant light conditions were consistently maintained across the photographing of all males; if a fish was slightly tilted more upward or downward, or even by turning the fish in order to photograph both its left and its right side, inconsistent results could have been

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[14] This last statement is somewhat reminiscent of notorious 'group selection' *sensu* Wynne-Edwards.

acquired due to changes in said angle (reflection of residual water may also play a part). In spite of this assertion, differences found were sooner significant between lines of selection than between replicates. This finding is contrary to Brooks and Endler (2001), who found female preference for iridescent area, or in other words found it to be an intersexually selected trait.

Dominant males had significantly longer tails than attractive males, yet the same result was not found for body length nor for total length. This is in concordance with Karino and Kobayashi (2005), who found that males with shorter tails were more attractive (female preference), and males with longer tails attempted more forced copulations (the more dominant strategy, attempting to circumvent female choice).

Alternatively, two studies show evidence in the opposite direction. Brooks and Endler (2001) found a positive correlation between female preference and tail area (tail area and tail length being naturally correlated; Appendix A), but similar to the study here, no such correlation was found for body area and female preference. Bischoff *et al.* (1985) also found a preference for larger tail area, in an experiment where they performed dichotomous choice trials between males with large and conspicuous tails, and males with shorter and less conspicuous tails. They even experimentally removed portions of conspicuously long tails for comparison. They also found that longer tailed males performed more displays. Seemingly, their results do not fully agree with the results here, since here the dominant males possessed longer tails, and these dominant males did not perform more displays and were unpreferred by females (5.1 Female choice)[15].

However, Karino and Matsunaga (2002) found that the female preferences reported are related to total length, rather than tail length. Similarly, Reynolds and Gross (1992) also found a female preference for male total length. Brooks and Endler (2001) did not look for effects of total length, they only performed their composite trait analyses on traits relating to colour (e.g.: contrast, etc.). Similarly, Bischoff *et al.* (1985) ignored body length and total length completely, their study has no mention of any correction relating to body size or even colouration for that matter. In fact, they did not strictly measure tail area, but rather subjectively categorised tail areas into two categories (conspicuous and inconspicuous). Consequently, both studies' results on tail area may have been confounded by total fish size

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[15] Nevertheless, a direct test of this assertion is lacking here. Since all of the experiments conducted were done separately, and as such the identities of males were not kept across experiments.

and overall male quality, which the females may have been preferring instead of the larger tails. This would also explain the higher display rates found by Bischoff *et al.* (1985), if longer tailed individuals represented larger and fitter individuals overall, these higher quality males might indeed perform substantially more displays. The unpreferred dominant males in this study did not have an increased total length in spite of their longer tails. It is thus hypothesised that increased tail lengths in male guppies may constitute a *badge of status* function in dominance.

5.5 On sexual conflict through sex ratio

In the following section, I would like to postulate a possible new avenue for research in sexual selection in guppies.

> *'If we consider the aggregate of an entire generation of such offspring it is clear that the total reproductive value of the males in this group is exactly equal to the total value of all the females, because each sex must supply half the ancestry of all future generation of the species. From this it follows that the sex ratio will so adjust itself, under the influence of Natural Selection, that the total parental expenditure incurred in respect of children of each sex, shall be equal; for if this were not so and the total expenditure incurred in producing males, for instance, were less than the total expenditure incurred in producing females, then since the total reproductive value of the males is equal to that of the females, it would follow that those parents, the innate tendencies of which caused them to produce males in excess, would, for the same expenditure, produce a greater amount of reproductive value; and in consequence would be the progenitors of a larger fraction of future generations than would parents having a congenital bias towards the production of females. Selection would thus raise the sex ratio until the expenditure upon males became equal to that upon females. [...] whichever [sex] is the scarcer, will be the more valuable, and consequently a more intense selection will be exerted in favour of all modifications tending towards its preservation.'*
> *~ Ronald Aylmer Fisher, The genetical theory of natural selection (1930)*

In the above abstract taken from Fisher, he described fluctuations in sex ratios due to differential mortality rates for both sexes at birth, subsequently a change in parental expenditure of the sexes is selected for. He thus attributed natural selection, rather than sexual selection, to fluctuations in sex ratio. It is, however, not inconceivable for sexual selection to potentially underlie such fluctuations as well (Kvarnemo and Ahnesjö, 1996). Where natural selection exerts its pressure on the parental expenditure, it can be argued that sexual selection creates differential potential reproductive values (or potential reproductive rates; PRR).

I would like to argue that in the guppy, sexual selection exerts pressure on alternate male mating strategies and brings about fluctuations in sex ratio, by the frequency dependent success of alternate male mating strategies (Gross, 1996). On the one hand, for instance, the more dominance oriented mating strategy (intrasexually driven) of forced and sneaky matings, and attempting to monopolise females; is likely more effective under a male-biased sex ratio. When relatively more males are present, male-male competition increases in intensity. Consequently, it decreases the potential of courting males to achieve consensual mating, because the increased intrasexual competition will incline to override female choice and male courtship (Bruce and White, 1995; Reichard *et al.*, 2005). Since the reproductive value of such a dominance strategy then rises relative to its alternative, it is probable that these males will benefit from biasing the sex ratio themselves. On the other hand, the strategy pertaining to female mate choice and courtship (intersexually driven) should favour either an unbiased or a more female biased sex ratio. This type of sex ratio decreases the odds of courtship being interrupted, in addition to attractive males having increased opportunities to attract more females. As such, each strategy's reproductive value is a function of the prevailing sex ratio in the population. Again, it should be pointed out that these behavioural strategies are not strictly discrete entities as described here (this is only done so for the sake of argument), they are continuous and not mutually exclusive. From a genetic perspective (*sensu* Dawkins, 1976), if these strategies bear a genetic bases (which is likely: Evans, 2010; Řežucha and Reichard, 2014) it is in their best interest to be genetically coupled to sex ratio distorting genes.

In addition to both strategies counterbalancing one another, each is counter selected by natural selection, towards an equal ratio. The fact remains that the scarcer gender will be selected for once it becomes underrepresented (see the citation above; Fisher, 1930), because each gender's reproductive value is ultimately also a function of its relative prevalence or scarcity.

Evidence of sex ratio distortion by males exists in guppies. Karino *et al.* (2006a) found that increasing male tail length biased sex ratios in favour of males. Due to the previous observation (Karino and Matsunaga, 2002) that female preference is related to total length rather than tail length (see also 5.4 Male morphology), they proposed that: '*[...] male guppies developed long tails as a deceptive tactic to attract females; this helps males in achieving large total lengths with lower growth investments when compared with that required to produce longer bodies*.' With this in mind, they considered their observation to be in line with the *attractiveness hypothesis* (Cockburn *et al.*, 2002), where: '*[...] females can enhance the* *fitness of their offspring by mating with an attractive partner by virtue of genes for viability and attractiveness, they overproduce sons if they can mate with an attractive male, but produce daughters when paired to an unattractive male.*'

As such, males with preferred longer tails leading to deceptively longer total lengths would constitute dishonest signalling, while generally honest signalling has been considered a prerequisite of any evolutionary stable strategy since Zahavi's (1975) paper. Dawkins and Guilford (1991) argue that corrupted signalling may exist, but only when there is a high enough cost incurred in receiver assessment of signals (the receiver in this case being the female). To my knowledge, no studies have been conducted regarding the costs of a female's assessment of males in guppies. Additionally Karino *et al.* (2006b) did find that choosing longer tailed males incurred a cost in females by means of a lower (total) reproductive output, so even if there were a female cost to assessment of signals, that cost would be heavily outweighed by the reduced reproductive output. Moreover, their earlier work seemingly undermines the '*longer tails as a deceptive attraction strategy*' as well, since they (Karino and Kobayashi, 2005) found that males with longer tails attempted more forced copulations on average, rather than attempting to attract females using their 'deceptive' total lengths. As such, Karino *et al.*'s (2006a) assertion apparently falls to pieces, and I suggest it to be a misinterpretation.

More in line with the experiments at the Tokyo Gakugei University (Karino and Matsunaga, 2002; Karino and Kobayashi, 2005; Karino *et al.*, 2006a, b), is the hypothesis postulated in this section. That is that intra- and intersexual selection might interact in guppies via sex ratio conflict, through the sex ratio dependent success of alternate mating strategies (male courtship versus male dominance and forced copulation). Dominant males with longer tails attempt to bias the sex ratio in favour of males, whereas attractive males might seek to keep an equal sex ratio or bias it towards females. Strands of evidence pointing in this direction are the combined results of: this study, where dominant/intrasexually selected males were found to have longer tails; the Karino *et al.* (2006a) study, where it was found that offspring sex ratio relates to male tail length; as well as the Karino and Kobayashi (2005) study, that found that males with longer tails perform more forced copulations.

Additionally there is circumstantial evidence in this study, concerning the sample sizes. Male sample sizes were highly skewed for the selection lines ($N_{attr,a}$=8, $N_{attr,b}$=16, $N_{dom,a}$= 16 and $N_{dom,b}$=29). This has no direct corollary with a sex ratio skew (brood sizes may have simply differed; no information on the number of females was recorded), but may serve as an interesting indication nonetheless. I propose that sex ratios of offspring could be recorded for each family unit (3.1 Species and husbandry) in the further maintenance of the artificial selection lines, to allow for a more direct test of this assertion. Overall, it is predicted that tail length relates to both male mating strategy and sex ratio distortion alike in guppies. In any case, more research is required with respect to tail length in guppies, to help further clarify the differences found in between the studies previously mentioned (i.e.: Bischoff *et al.,* 1985; Brooks and Endler, 2001 versus; Karino and Matsunaga, 2002; Karino and Kobayashi, 2005; Karino *et al.,* 2006a, b). Future research might also be directed at the relationship between dominance and tail length, to directly test the possible *badge of status* function of long tails in guppies.

6 Conclusion

It is shown that inter- and intrasexual selection both select for alternate mating strategies in male guppies, and that even though male guppies can adapt their behaviour and adopt more than one strategy, there may be a genetic basis for inherent preferred mating strategies (e.g. attractive males and showiness). Female preference was not swayed, neither were relevant differences in the female reproductive behavioural phenotype found. Further artificial selection, through more generations, may be necessary to bring this about. Rare males were preferred by females with regard to orange colouration, in concordance with negative frequency dependent selection. Preference may yet lie in colour chroma and brightness as well. Intrasexually selected males showed potential for increased iridescence colouration. No increased aggression was found for males adopting the more dominant strategy, although a multi-male set-up may be needed to verify this. Tail lengths are likely to be a *badge of status* for males in male-male competition. Overall, intra- and intersexual selection do not act in concert in the guppy (*Poecilia reticulata*), for male reproductive behaviour and male morphology alike. This discrepancy is argued to be based in the absence of territoriality, paternal investment and a resource system in the guppy mating system; leading to a lack of direct benefits for mating with dominant males. It is further proposed that intra- and intersexual selection might interact in guppies via sex ratio conflict, through sex ratio dependent success of alternate mating strategies.

7 Acknowledgements

I would like to extend my thanks to my promotor Dr. Katleen Huyghe, for her advice, her help in setting up the experiments, maintaining the guppies and selection lines, for her inexhaustible patience and for entrusting me with her set-up. Moreover, I would like to express my gratitude towards my co-promotor Prof. Dr. Raoul Van Damme for his help and support, Mr. Jan Scholliers for his help in creating the experimental set-ups (and aiding me during basement flooding scenarios), and the Phd. students Tess Driessens and Glenn Borgmans for their help in clarifying the Jwatcher program. Furthermore, I would like to express my appreciation towards Mrs. Eva Goethals and Mrs. Veerle Geuens of the University's student counselling for supporting me in my endeavors, and towards Dr. James Weedon (PLECO) and Prof. Dr. Kris Van De Poel (Applied linguistics) for both their encouragement and constructive criticisms on academic English writing. Lastly, I would like to thank Mrs. Margaret Lindholm unaware of her own help in reminding me that; the weakest has but to try his strength to find it, and then he shall be strong.

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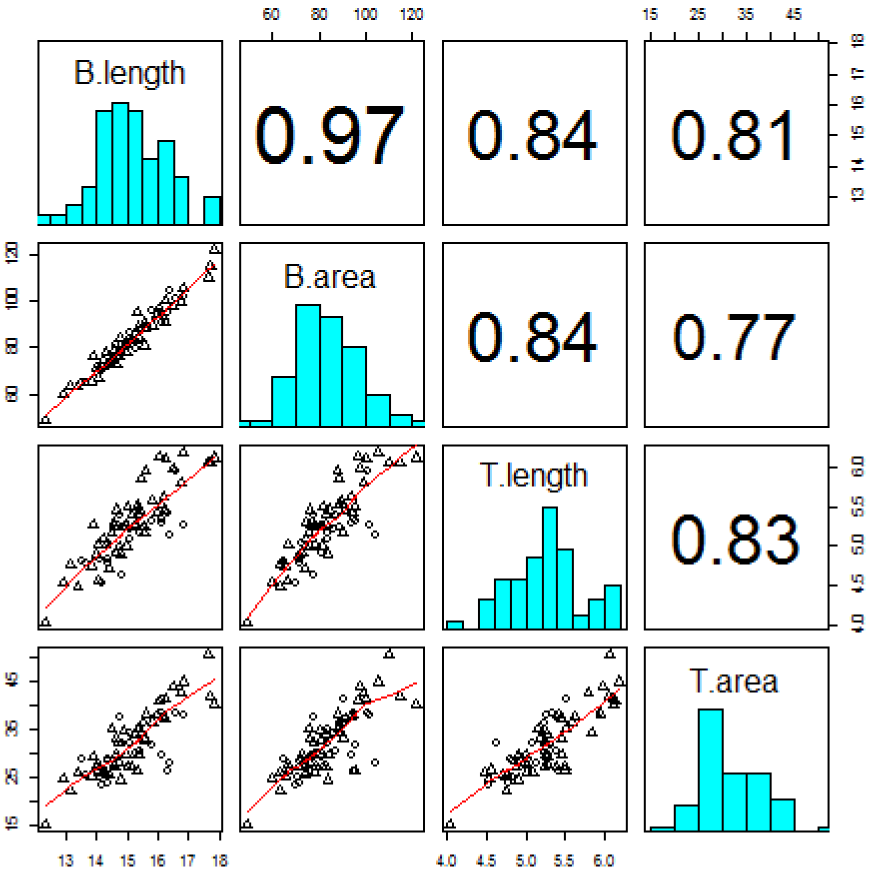
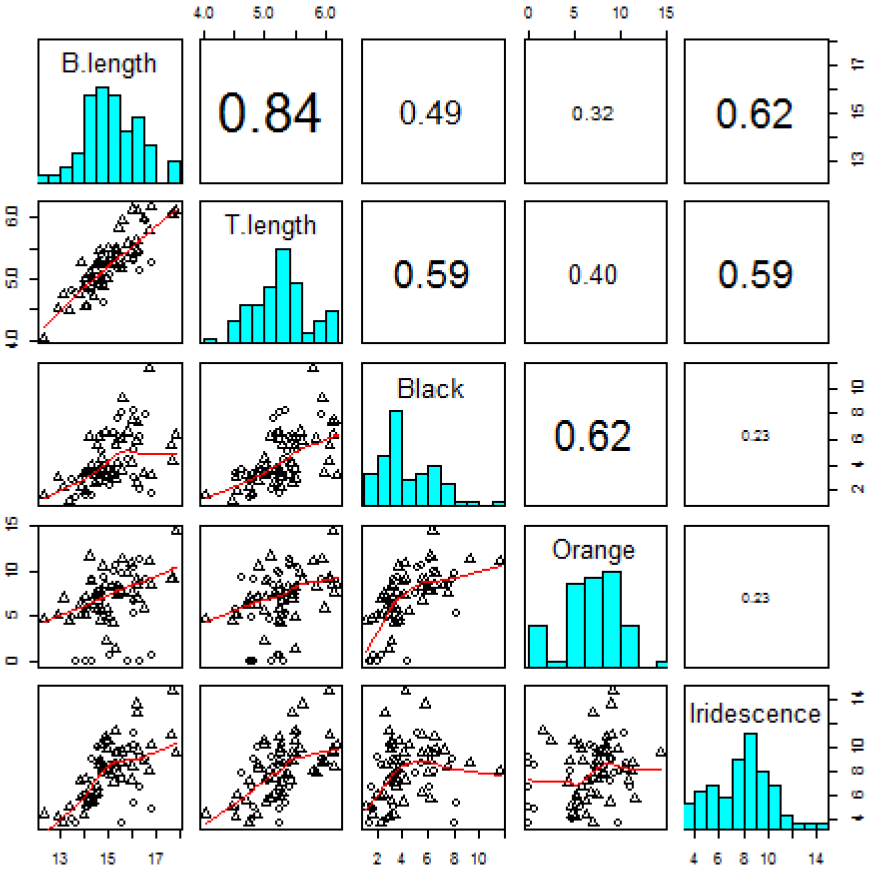
9 Appendices



Appendix A: Correlations among male guppy morphometrics, each square depicts a correlation between the indicated trait of row and column. With; *B.length* = average body length, *B.area* = average body area, *T.length* = average tail length and *T.area* = average tail area. Averages were taken from only two measurements (left and right side photograph) for each guppy (in mm for lengths and mm^2 for surfaces). The upper panel shows absolute correlations for each pair of traits, with its size being proportionate to the strength of correlation.



Appendix B: Correlations among male guppy morphometrics as well as measurements of colour, each square depicts a correlation between the indicated trait of row and column. With; *B.length* = average body length (mm), *T.length* = average tail length (mm), and the three colours whose surfaces were quantified (mm^2). Averages were taken from only two measurements (left and right side photograph) for each guppy (in mm for lengths and mm^2 for surfaces). The upper panel shows absolute correlations for each pair of traits, with its size being proportionate to the strength of correlation.