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Journal:	*Parasitology*
Manuscript ID:	PAR-2015-0174.R1
Manuscript Type:	Research Article - Standard
Date Submitted by the Author:	08-Aug-2015
Complete List of Authors:	Dargent, Felipe; McGill University, Biology Reddon, Adam; McGill University, Biology Swaney, William; Liverpool John Moores University, School of Natural Sciences and Psychology Fussmann, Gregor; McGill University, Biology Reader, Simon; McGill University, Biology Scott, Marilyn; McGill University, Institute of Parasitology Forbes, Mark; Carleton University, Biology
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Title

Demasculinisation of male guppies increases resistance to a common and harmful ectoparasite

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Running title

Demasculinisation and ectoparasite resistance

SUMMARY

Parasites are detrimental to host fitness and therefore should strongly select for host defence mechanisms. Yet, hosts vary considerably in their observed parasite loads. One notable source of inter-individual variation in parasitism is host sex. Such variation could be caused by the immunomodulatory effects of gonadal steroids. Here we assess the influence of gonadal steroids on the ability of guppies (*Poecilia reticulata*) to defend themselves against a common and deleterious parasite (*Gyrodactylus turnbulli*). Adult male guppies underwent 31 days of artificial demasculinisation with the androgen receptor-antagonist flutamide, or feminisation with a combination of flutamide and the synthetic oestrogen 17β-estradiol, and their parasite loads were compared over time to untreated males and females. Both demasculinised and feminised male guppies had lower *G. turnbulli* loads than the untreated males and females, but this effect appeared to be mainly the result of demasculinisation, with feminisation having no additional measurable effect. Furthermore, demasculinised males, feminised males and untreated females all suffered lower *Gyrodactylus*-induced mortality than untreated males. Together, these results suggest that androgens reduce the ability of guppies to control parasite loads, and modulate resistance to and survival from infection. We discuss the relevance of these findings for understanding constraints on the evolution of resistance in guppies and other vertebrates.

Key words

gonadal steroids, feminisation, sex-biased parasitism, testosterone, fish, *Gyrodactylus, Poecilia reticulata*

Key findings

- Blockage of androgen receptors led to lower ectoparasite loads in male guppies
- Additional treatment with oestrogen did not reduce parasitism further
- Treated males experienced *lower* parasite-induced mortality than untreated males

INTRODUCTION

Parasites are pervasive and are known to negatively influence host fitness by reducing reproductive output, growth rate, mating success, and survivorship (Price, 1980). In doing so, parasites can be influential drivers of ecological processes and evolutionary patterns (Hamilton, 1982; Hamilton & Zuk, 1982; Lafferty *et al.*, 2008; Minchella & Scott, 1991). Parasitism is expected to be a strong source of selection for defensive adaptations that allow hosts to control parasite numbers and mitigate parasite costs. When parasites are present, investment in costly defence mechanisms is expected to be favoured (Schmid-Hempel, 2011). Intriguingly, there is considerable within-population variation amongst individuals in their susceptibility to parasites, suggesting that antiparasite defences are costly and/or trade-off with other fitness enhancing traits, and therefore that maximal defence may not be obtainable or adaptive for all individuals (Lazzaro & Little, 2009; Sheldon & Verhulst, 1996). A striking example of among-individual variation in parasite susceptibility is the common phenomenon of sex-biased parasitism, in which one sex is more frequently infected or carries larger mean parasite loads than the other (Forbes, 2007; Krasnov *et al.*, 2012; Nunn *et al.*, 2009; Zuk & McKean, 1996). For example Amo *et al.* (2005) found that wild male wall lizards (*Podarcis murallis*) had higher haemogregarine and ectoparasitic mite infection intensities than did females. Similarly, Krasnov *et al.* (2005) found higher flea abundance in males than females of six out of nine species of desert rodent.

Males and females differ in many ways that may partially account for sex differences in parasite infection rates. For example, males and females often differ in body size and larger individuals typically have more parasites (Guégan *et al.*, 1992; Poulin & Rohde, 1997). Males and females may also be exposed to parasites at different rates due to sex differences in space use or social behaviour (Tinsley, 1989). Furthermore, sex differences in time and energy

allocation to sexual activities (e.g. courting and fighting) and resource acquisition also could drive sex differences in parasite loads through differences in the amount of resources available for investment in defence (Zuk, 1990).

Gonadal steroids play a critical role in sexual differentiation during development, resulting in sex differences in anatomy, physiology, and behaviour (Arnold, 2009; Wallen & Baum, 2002), and therefore may have a long-term influence on sex-biased parasitism by organizing phenotypic characteristics during development which in turn affect parasite defence later in life. However, gonadal steroids also can have a more immediate influence on sex-biased parasitism because variation in circulating hormones in adults can mediate sex differences in immune function (Grossman, 1989; Zuk & McKean, 1996).Understanding precisely how circulating gonadal steroids influence defence is a crucial step in understanding individual variation in defence, as well as the potential for and magnitude of sex-biased parasitism, which in turn are necessary for understanding host-parasite dynamics in natural systems. To this end, it is essential to evaluate both the role of gonadal steroids during development and the role that circulating gonadal steroids play in parasite resistance in adults.

Here, we studied guppies (*Poecilia reticulata*) derived from wild populations and their common and harmful ectoparasites (*Gyrodactylus turnbulli*) to address the importance of circulating gonadal steroids in determining antiparasite defences, i.e. the effect that steroid hormone systems have on adult resistance to parasites. To this end we manipulated gonadal steroid levels in adult guppies by administering an androgen receptor antagonist (to demasculinise them), or a combination of an androgen receptor antagonist and an artificial oestrogen (to demasculinise and then feminise them), before assessing their resistance to *G. turnbulli*.

MATERIALS AND METHODS

The study system

The guppy is a sexually-dimorphic poeciliid fish native to the island of Trinidad and Northern South America (Magurran, 2005). *Gyrodactylus turnbulli* is a highly prevalent (Harris & Lyles, 1992) and deleterious ectoparasite of wild guppies (Gotanda *et al.*, 2013; van Oosterhout *et al.*, 2007a). These monogenean flatworms transmit through host-to-host contact, and attach to their host's epithelium where they feed and give birth to flukes with fully developed embryos "in-utero" (Bakke *et al.*, 2007). Therefore, *Gyrodactylus* infections are prone to exponential population increase on individual hosts and epidemic dynamics within guppy populations (Scott & Anderson, 1984), which leads to high guppy mortality in the laboratory (Dargent *et al.*, 2013a; Van Oosterhout *et al.*, 2007b) and the wild (van Oosterhout *et al.*, 2007a).

The guppy-*Gyrodactylus* host-parasite system is a convenient model to assess the role of gonadal steroids in the defence against parasites. First, the regulatory effect of androgens on guppy behaviour and colouration may play a critical role in the expression of secondary sexual characters and mating success (Bayley *et al.*, 2002; 2003). Second, correlations between carotenoid colouration, mate preference and defence against parasites have long been recognised in guppies (Houde & Torio, 1992; Kennedy *et al.*, 1987; Kolluru *et al.*, 2006) while the ecological and evolutionary drivers of guppy parasite defence have been the focus of much recent research (Dargent *et al.*, 2013a; Dargent *et al.*, 2013b; Fitzpatrick *et al.*, 2014; Gotanda *et al.*, 2013; Perez-Jvostov *et al.*, 2012; Pérez-Jvostov *et al.*, 2015; Tadiri *et al.*, 2013). Missing from this increasingly well-understood model system is the degree to which circulating gonadal steroids influence defence against *Gyrodactylus* parasites in the guppy.

Guppies used in this research were laboratory-reared from fish collected in Trinidad. In Experiment 1, we used F2 descendants from a Trinidadian population collected in 2013 after having been experimentally translocated in 2009 (Travis *et al.*, 2014) from a high-predation site in the Guanapo river where *Gyrodactylus* spp. was present to a tributary stream (Lower Lalaja) where predation was low and *Gyrodactylus* was absent. In experiment 2, we used F1 descendants of wild-caught fish collected between 2010 and 2011 from the Aripo and Quare rivers from sites where predation is high and *Gyrodactylus* spp. is present. These guppies were kept together as a mixed origin population.

Hormone treatments

Two weeks prior to the start of the hormone treatments, sexually mature guppies were isolated into 1.8 L chambers in an aquatic housing system (Aquaneering Inc., San Diego, USA). The fish were physically isolated but retained visual contact with their neighbours throughout the experiments. The laboratory was maintained at 23 ±1°C with a 13 h 11 h (L:D) photoperiod. We used carbon-filtered municipal water that was conditioned with Prime (Seachem Laboratories , Madison, USA), Freshwater Biozyme (Mardel, Oklahoma city, USA), and left to stand for two days and warm up before being added to the housing systems. The housing system passed water through a filter pad, a biological filter, a set of carbon filters and a UV sterilization device. Subjects were fed with TetraMin Tropical Flakes (Tetra, Melle, Germany) ground into powder and reconstituted with water to form a thick paste that was delivered using Hamilton microliter syringes (Hamilton Laboratory Products, Reno, USA). Prior to the start of the hormone treatments subjects were fed *ad libitum* and their chambers remained connected to the

138 re-circulating system, thus each chamber had a complete water change approximately every 8
139 minutes.

140 We gathered data on individual body size (measured as standard length: SL) and mass at
141 two time points: on the first day we began administering the hormone treatments, and 21 days
142 later, the day we began experimental parasite infections (Tables S1, S2). To measure SL and
143 mass we anesthetised each guppy in 0.02% Tricaine Methanesulfonate - MS222 (Argent
144 Chemical Laboratories, Redmond, USA) buffered to a pH of 7 with $NaCO_3$. Guppies were then
145 weighed to the nearest 0.01 g and photographed on the left side with a Nikon D90 camera
146 (Nikon, Mississauga, Canada). Each image included a ruler for scale.

147 At the start of the hormone treatments, male guppies (mean mass = 0.08 g ±0.002 s.e.m.)
148 were randomly assigned to control, demasculinisation or feminisation treatments, while females
149 (mean mass = 0.13 g ±0.006 s.e.m.) remained untreated. Acetone was used as a solvent to
150 combine the pharmacological agents with ground flake food. We saturated the food with acetone
151 mixed with the hormone treatment and then allowed the acetone to evaporate in a fume hood for
152 24 hours. Untreated control male and female guppies received food that had been saturated with
153 acetone alone without any pharmacological treatment, guppies in the demasculinisation
154 treatment received food that had been dosed with 4.29 mg of the androgen receptor antagonist
155 flutamide (Sigma-Aldrich, Oakville, Canada) per gram of dry food, and guppies in the
156 feminisation treatment received food that had been dosed with 4.29 mg of flutamide and 0.04 mg
157 of the synthetic oestrogen 17β-estradiol (Sigma-Aldrich, Oakville, Canada) per gram of dry food.
158 Each guppy received 5 µL/day of paste prepared with their respective treatments (in a 7:8
159 food:water ratio), which is equivalent to 10.40 µg/day/guppy of flutamide and 0.10 µg/day/guppy
160 of 17β-estradiol. Guppies ingested all of the food provided to them. The flutamide dosage was

based on previous dose-response studies in guppies showing effective inhibition of male-specific traits (Bayley *et al.*, 2003; Kinnberg & Toft, 2003), without the increased mortality seen at higher doses (Baatrup & Junge, 2001). The dose of 17β-estradiol/g body weight was based on dose-response work in goldfish demonstrating robust inhibition of male-specific traits, but no associated weight loss (Bjerselius *et al.*, 2001). All hormone treatments lasted for 31 days (i.e. 21 days of treatment without parasite infections and 10 days of treatment after *Gyrodactylus* infection). We performed two consecutive experiments. Experiment 1 had two treatments: feminisation of males and untreated males. Experiment 2 had the same treatments as Experiment 1 in addition to demasculinisation of males and untreated females. These experiments were identical in all regards with the exception of the additional treatments (see below) and the use of different wild-derived guppy populations.

During the 31 days of hormone treatment, the guppies remained in their 1.8 L chambers, which we disconnected from the aquatic recirculating system, chemically isolating the fish to ensure that no hormone treatment passed between the chambers. Visual contact between neighbours was retained throughout the experiment and therefore the fish were not socially isolated at any time. To maintain water quality during the treatment period, we changed 75% of the water in each chamber every four days and replaced the chamber with an entirely fresh one every 12 days. Water quality was monitored throughout the experiments by performing visual checks for water clarity and residue presence and by weekly tests, in randomly selected chambers, of alkalinity, pH, nitrite, nitrate, hardness and ammonia . Water quality was within normal range throughout and we did not detect any sign of water quality degradation at any time, or of negative effects of water quality on the hosts or parasites.

184 *Experimental Infections*

185 21 days after the start of the hormone treatments, all fish were individually anaesthetised in
186 0.02% MS222 and infected with two *Gyrodactylus turnbulli* each. We infected each guppy by
187 removing a small piece of fin tissue or a scale carrying *G. turnbulli* from a euthanized infected
188 donor guppy and placing it next to the recipient guppy's caudal fin until we saw, under a Nikon
189 SMZ800 dissecting stereoscope (Nikon Instruments, Melville, USA), that two *G. turnbulli* had
190 attached to the experimental fish. After infection, each guppy was allowed to recover from
191 anaesthesia in its home chamber. We monitored *G. turnbulli* numbers on each live subject on
192 days 6, 8 and 10 post infection, by anaesthetising the fish and counting the parasites using the
193 dissecting stereoscope at 18x magnification. We used *G. turnbulli* from our laboratory
194 population, which was initially obtained in 2009 from domestic guppies purchased from a
195 commercial supplier in Montreal, QC, Canada. This *G. turnbulli* population has been maintained
196 on domestic-origin host guppies, and therefore has not had any period of coevolution with the
197 wild-origin guppy populations used in this study.
198
199 *Analysis*
200 To assess whether hormone treatment and guppy body size (SL) had an effect on *G. turnbulli*
201 load on each count day, we fitted a generalised linear model (GLM) with a negative binomial
202 distribution and a log link function using Tukey HSD for pairwise *post-hoc* comparisons. To
203 assess the general effect of hormone treatment on the growth trajectory of *G. turnbulli* we fitted a
204 repeated measures GLM with a negative binomial distribution for Experiment 1. We were unable
205 to perform this analysis for Experiment 2 because of the high parasite-induced mortality in the
206 untreated control group. The repeated measures GLM was conducted in SPSS 22 (IBM, New

York, USA), all remaining analyses were conducted using the R Language and Environment for Statistical Computing v 3.1.0 (R Development Core Team, 2014). α was set at $p<0.05$. Data are archived in the Dryad repository (link to be added).

Experiment 1

To assess whether guppy resistance was influenced by the action of circulating gonadal steroids we compared 15 untreated males to 14 males that had been treated simultaneously with flutamide (an androgen receptor antagonist) and 17 β-estradiol (a synthetic oestrogen) (Table S3). Guppy body size and mass did not significantly differ between treatments (feminisation vs. untreated) at the start of the experiment (SL: $F_{1,27}=0.91$, $p=0.35$; mass: $F_{1,27}=0.23$, $p=0.63$), nor at the start of infection (i.e., 21 days after the start of hormone treatment; SL: $F_{1,26}=0.14$, $p=0.71$; mass: $F_{1,27}=0.01$, $p=0.91$). Subjects were laboratory-reared F2 descendants from a Trinidadian population experimentally translocated in 2009 (Travis *et al.*, 2014).

Experiment 2

To disentangle the relative roles of androgens and oestrogens, we ran a second experiment, repeating both treatments in Experiment 1 along with two additional treatments: male demasculinisation and untreated females, resulting in four total treatment groups (Table S4). Males under demasculinisation were treated with flutamide only, allowing us to investigate male parasite resistance when androgen receptor signalling is blocked. Different gonadal steroids can have contrasting effects on immune function: androgens generally have immunosuppressive effects, while oestrogens often promote disease resistance, although effects can vary (Klein, 2000; 2004). We also tested untreated females to check for sex-biased parasite resistance in

untreated guppies, as this is known to vary between populations (Dargent, 2015; Gotanda *et al.*, 2013; Stephenson *et al.*, 2015).

As is typical for guppies, the females were larger than the males, both at the beginning of the experiment (mean ± s.e.m. SL: males=15.46 ±0.16, females= 17.97 ±0.3; $F_{3,65}$=21.28 , p<0.001) and at the time of infection (mean ± s.e.m. SL: males=15.56 ±0.14, females=18.57 ±0.28; $F_{3,68}$=36.99, p<0.001). There was no significantdifference in SL among the three male treatments at either time point (start of treatments: $F_{2,47}$=1.1, p=0.34; infection: $F_{2,50}$=0.72, p=0.49). A similar pattern was observed for body mass. Female guppies were heavier than males when they started receiving the hormone treatments (mean ± s.e.m.: males=0.09 ±0.003, females=0.13 ±0.006; ($F_{3,65}$=14.73, p<0.001) and on the first day of infection (mean ± s.e.m.: males=0.08 ±0.003, females=0.14 ±0.006; $F_{3,68}$=31.17, p<0.001), but, mass did not differ between male treatments at the start of the experiment ($F_{2,47}$=0.38, p=0.68) nor on the day of infection ($F_{2,50}$=0.24, p=0.79). Males did not differ in SL between Experiment 1 and 2 (initial SL: $F_{1,77}$=0.42, p=0.52; infection day SL: $F_{1,79}$=0.004, p=0.95) but males in Experiment 1 were lighter than those in Experiment 2 (initial mass: $F_{1,77}$=6.21, p=0.01; infection day mass: $F_{1,80}$=4.53, p=0.04; Tables S1, S2).

Post-infection mortality was high in Experiment 2 and so we used a Cox proportional hazards model to determine whether hormone treatment and body size (SL) influenced guppy survival up to 13 days post infection (i.e. three days after we had finished treating the guppies with hormones). Standard length and its interaction with hormone treatment had no significant effects on survival and thus were dropped from the model by AIC step-wise model selection.

253 RESULTS

254 *Experiment 1*

255 Guppies that underwent feminisation via treatment with flutamide and 17β-estradiol had
256 significantly lower *G. turnbulli* loads than untreated guppies throughout the infection period
257 (repeated measures GLM effect of treatment: $F_{1,77} = 4.94$, $p < 0.029$), and specifically on both
258 Day 8 and Day 10 of infection (Table 1, Figure 1, Figure S5). We did not detect any significant
259 effect of SL or of its interaction with the hormone treatment on parasite load (Table 1). Mortality
260 following infection was low. Only 3 individuals had died (10%) by Day 10 of infection, all of
261 which were in the untreated group (Table S3). *G. turnbulli* populations on individual guppies
262 continued to grow through the duration of the experiment (Figure S5). We observed no obvious
263 pathological effects of treatment with flutamide and 17β-estradiol in concert (feminization), and
264 this treatment significantly increased resistance to *Gyrodactylus turnbulli* on all guppies.

265

266 *Experiment 2*

267 Hormone treatment had a significant effect on parasite load at both Day 8 and Day 10 (Table 2,
268 Figure 2, Figure S6). As in Experiment 1, males that underwent feminisation also had lower *G.*
269 *turnbulli* loads on both Day 8 and Day 10 of infection compared to untreated males (Tables 2, 3,
270 Figure 2), although this difference was only statistically significant on Day 10. Males that
271 underwent the demasculisation treatment had significantly lower *G. turnbulli* loads compared to
272 untreated males on Day 8 and 10 of infection (Tables 2, 3, Figure 2). Parasite loads were not
273 significantly different between those males that underwent demasculinisation and those that
274 underwent feminisation at any time point, and both had lower loads than untreated females on
275 Day 10 (Tables 2, 3, Figure 2). With few exceptions, *G. turnbulli* populations on individual

guppies continued to grow for the duration of the experiment while their hosts remained alive, but growth trajectories differed with treatments (Figure S6). We observed no significant effects of SL or any interaction effects between body size and treatment on parasite load (Table 2). Contrary to previous studies on wild guppy populations (Gotanda *et al.*, 2013), we found no evidence that guppies from our Aripo/Quare mixed-origin laboratory-bred population were sexually dimorphic in *G. turnbulli* resistance (Table 3). In contrast to Experiment 1, guppy mortality after infection with *G. turnbulli* was high in the mixed Aripo/Quare population: 67% of all fish had died by the 13th day of infection (56% by the 10th day). This mortality was significantly higher in the untreated males than in either group of treated males (demasculinisation or feminisation) or the untreated females (Tables 4, S2).

DISCUSSION

We conducted two independent experiments with different populations of wild-origin guppies and found that gonadal steroid affects the ability of male guppies to control infection by the ectoparasite *Gyrodactylus turnbulli. G. turnbulli* populations on individual hosts increased over the experiment, but treatment with the androgen receptor antagonist flutamide (resulting in 'demasculinised' males) or a combination of flutamide and the oestrogen 17β-estradiol (resulting in 'feminised' males) resulted in reduced *G. turnbulli* loads compared to untreated males or females. These differences were not explained by differences in body size. Furthermore, males under both feminisation and demasculinisation treatments showed significantly greater survival compared to untreated males following infection in our second experiment. Variation in *G. turnbulli* population growth within treatments and between experiments is likely to be influenced by the autocorrelative nature of *Gyrodactylus* population growth (Ramírez *et al.*, 2012), yet the

299 effects of gonadal steroid manipulation generated significantly different parasite loads between
300 treatments in both experiments. Taken as a whole, these results suggest that androgens have a
301 detrimental effect on guppy resistance to parasitism.

302 To our knowledge, only one previous study has experimentally assessed the role of
303 gonadal steroids on *Gyrodactylus* resistance. Buchmann (1997) evaluated the effect of
304 testosterone on female trout (*Oncorhynchus mykiss*) resistance to *Gyrodactylus derjavini* and
305 concluded that testosterone injections led to higher parasite loads. However, the results of the
306 Buchmann (1997) study could not distinguish between a detrimental effect of testosterone on
307 host defence and the alternative hypothesis that testosterone has a direct positive effect on
308 *Gyrodactylus* reproduction. Our results suggest that a detrimental effect of androgens on the host
309 is more likely than a direct effect of testosterone on *Gyrodactylus* reproduction. Our
310 experimental fish received flutamide, which binds to androgen receptors broadly inhibiting the
311 host physiological response to multiple androgens in teleost fishes (including both testosterone
312 and 11-ketotestosterone; de Waal *et al.*, 2008; Jolly *et al.*, 2006) without altering the circulating
313 levels of these hormones (Jensen *et al.*, 2004).

314 Oestrogens also may have immunomodulatory effects in teleost fishes (e.g. Cuesta *et al.*,
315 2007; Watanuki *et al.*, 2002), but the degree to which they enhance or reduce host immunity
316 seems to be highly system and species-specific (Chaves-Pozo *et al.*, 2012). When we consider
317 the role of oestrogens on defence against *Gyrodactylus*, two lines of evidence suggest that it did
318 not have a major effect in the guppy. First, male guppies treated with flutamide and 17β-estradiol
319 did not differ in resistance from male guppies treated with flutamide alone, suggesting that 17β-
320 estradiol did not have a substantial additional effect on defence. Second, untreated female

guppies were not more resistant than males that underwent demasculinisation and, in fact, they had higher parasite burdens on Day 10 of infection.

Female guppies are larger than males and sexual dimorphism in body size is a common explanation for sex-biased parasitism in vertebrates. The larger sex is expected to have higher parasite loads since larger individuals have a wider area exposed to parasite attacks or a larger resource base for the parasite population to grow (Zuk & McKean, 1996). However, we did not detect a difference in parasite loads between untreated males and females, nor did body size correlate with variation in resistance in either experiment. This finding might appear surprising, given that field surveys (Gotanda *et al.*, 2013) and laboratory experiments (Dargent, 2015) report sex differences in *Gyrodactylus* load in certain guppy populations, and in at least one instance such a sex difference has been linked to size dimorphism (Cable & van Oosterhout, 2007). However, sex-biased parasitism in guppies is not consistently male biased and appears to be influenced by predation regime. For example, Gotanda *et al.* (2013) reported higher *Gyrodactylus* spp. loads on females compared to males in natural streams where the risk of predation was high but the reverse pattern at sites where the risk of predation was low, suggesting that body size differences are not a comprehensive explanation for sex-biased parasite loads in guppies. Furthermore, experimentally translocated wild guppies have been shown to rapidly evolve resistance to *Gyrodactylus* in a sex-specific manner, leading to the loss of sexual dimorphism in resistance (Dargent, 2015). Thus, it is possible that the population we used for Experiment 2 is one that shows minimal sexual dimorphism in resistance to *Gyrodactylus*, although we did observe higher mortality in untreated males than females. It is possible that the high mortality in the untreated male group, which considerably reduced our sample size, precluded our ability to detect an otherwise significant dimorphism in parasite loads.

344 Nevertheless, our results clearly show that regardless of any initial sexual dimorphism in
345 defence, interfering with androgen signalling augments resistance to *G. turnbulli* in male
346 guppies.

347 The significantly higher mortality of untreated males compared to untreated females
348 suggests that androgens may reduce guppy tolerance to *Gyrodactylus* (i.e. the ability of hosts to
349 reduce the negative impacts of a given parasite load; Raberg et al. 2007). This line of reasoning
350 is supported by the lower mortality of males that underwent both demasculinisation and
351 feminisation compared to the untreated males. We did observe a difference in untreated male
352 mortality between Experiments 1 and 2, possibly the result of population differences in subjects'
353 susceptibility to *Gyrodactylus* induced mortality. Given that laboratory conditions were identical
354 between the two experiments, the most likely cause for particular differences in mortality and
355 parasite loads between Experiment 1 and Experiment 2 is the fish population of origin. However,
356 regardless of population origin, guppies that underwent hormone treatments (demasculinisation
357 or feminisation) experienced lower mortality during infection and carried lower parasite loads
358 than untreated males in both experiments.

359 The suppressive effect of the androgen system on guppy defence against the model
360 monogenean *G. turnbulli* suggests a trade-off between resistance to these ectoparasites and other
361 fitness-related traits of male guppies. On the one hand, female guppies typically prefer to mate
362 with males that show brighter carotenoid colouration (Houde & Endler, 1990) and more active
363 courtship (Kodric-Brown & Nicoletto, 2001), traits which have positive correlations with
364 circulating androgens (Baatrup & Junge, 2001; Bayley *et al.*, 2003), and thus higher levels of
365 circulating androgens would seem to increase male fitness. On the other hand, infections by
366 *Gyrodactylus* are known to decrease male carotenoid colouration and display rate, and

consequently decrease female preference for males with higher *Gyrodactylus* loads (Houde & Torio, 1992; Kennedy *et al.*, 1987). Furthermore, *Gyrodactylus* infection may compromise predator evasion, for example via increased morbidity and decreased swimming performance (Cable *et al.*, 2002). Thus *Gyrodactylus* can decrease male guppy host fitness through the direct effect of increased mortality and through the indirect effect of decreased mating opportunities, which may counterbalance the fitness enhancing properties of their androgen hormones. A further possibility is that increases in circulating androgens could promote carotenoid accumulation, that in turn counterbalances the immunosuppressive effects of androgens (e.g. Blas *et al.*, 2006; McGraw & Ardia, 2007). However, this possibility is unlikely here, given that males with intact androgen levels had higher parasite burdens than those under the feminisation and demasculinisation treatments.

In conclusion, a reduced response of androgen receptors to circulating androgens was found to lead to decreased parasite burdens and parasite-induced mortality. Future work should determine if androgens have a direct immunosuppressive effect or, alternatively, if androgen dependent changes in sexual traits and reproductive investment indirectly affects investment in immunity. Our findings are consistent with the idea that androgens modulate immune function but run contrary to the view that size determines parasite loads, and therefore help further the understanding of inter-individual variation in parasitism. The developmental and current (circulating) effects of gonadal steroids on the immune system and resistance to infection, as well as their indirect effects on secondary sexual traits that affect fitness, are underappreciated in studies addressing the ecology and evolution of vertebrate defence against parasites. Our results on a model host-parasite system strongly suggest that gonadal steroids should be considered in

concert with morphological or behavioural differences when accounting for variation among individuals and between the sexes.

ACKNOWLEDGEMENTS

We thank HJ Pak, K White, D Uthayakumar and G Daggupati for laboratory assistance, A Morrill and S Portalier for figure coding advice, and A Hendry for use of his aquatic housing systems (NSERC-RTI #148297). We thank D Reznick, C Ghalambor, E Ruell, D Fraser, and the FIBR team for supplying us with guppies used in Experiment 1.

FINANCIAL SUPPORT

We thank the Quebec Centre for Biodiversity Science (FD); the Natural Sciences and Engineering Research Council of Canada (NSERC) (GFF - #356373-07; SMR - #418342-2012 and #429385-2012; ARR), Richard H. Tomlinson fund (ARR) and the Canada Foundation for Innovation (SMR - #29433). Research at the Ghalambor lab was supported by a NSF Faculty Early Career (DEB-0846175). The guppy introductions were funded by a United States NSF-Frontiers in Integrative Biological Research grant to D Reznick P.I. (EF-0623632).

ETHICAL STATEMENT

This study was carried out in accordance with the regulations of the McGill University Animal Care Committee (AUP #5759) and the guidelines of the Canadian Council on Animal Care.

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Legends to figures

Figure 1: Mean *Gyrodactylus turnbulli* parasite load per host for untreated male guppies (dashed line) or males treated with flutamide and 17β-estradiol (feminisation - solid line) by day of infection (Experiment 1).

Figure 2: Mean *Gyrodactylus turnbulli* load per host in male guppies treated with flutamide (demasculinisation), with flutamide and 17β-estradiol (feminisation), and in untreated males and females, compared across days after infection (Experiment 2). Points are slightly offset on the x axis to reduce overlap.

632 Tables

633 **Table 1:** *Gyrodactylus turnbulli* parasite load is significantly higher in untreated male guppies
634 compared to males under feminisation on Day 8 and 10 of infection (Experiment 1).

635

	Day 6[a]	Day 8[b]	Day 10[c]
Hormone treatment	3	5.49*	5.01*
SL	2.73	2.44	1.18
Treatment:SL	0.83	0.35	0.12

636 [a] n=29; [b] n=28; [c] n=26.

637 Generalized linear model results for *Gyrodactylus turnbulli* load (integer variable with a negative
638 binomial distribution) on guppies for days 6, 8 and 10 of infection, with treatment (feminisation
639 vs. untreated males) as factor and guppy standard length (SL) as a covariate. F-values reported,
640 significant differences in bold (*=$p<0.05$).

641

642 **Table 2:** *Gyrodactylus turnbulli* parasite load varies with sex and hormone treatment on Day 8
643 and 10 of infection (Experiment 2).

	Day 6[a]	Day 8[b]	Day 10[c]
Hormone treatment	2.5	3.26*	11.25***
SL	0.81	0.26	0.29
Treatment:SL	0.49	0.24	2.68

644 [a] n=72; [b] n=62; [c] n=40.
645 Generalized linear model results for *Gyrodactylus turnbulli* load (integer variable with a negative
646 binomial distribution) on guppies for days 6, 8 and 10 of infection, with treatment (untreated
647 males, untreated females, males under demasculinisation, and males under feminisation) as
648 factor and guppy standard length (SL) as a covariate. F-values reported, significant variables in
649 bold (*=p<0.05, ***=p<0.001).
650
651

652 **Table 3:** *Post-hoc* pairwise comparisons of *Gyrodactylus turnbulli* load by treatment

653 (Experiment 2)

Treatment pair	Day 8		Day 10	
	diff.	adj. p	diff.	adj. p
UF-UM	-19.25	0.24	-80.15	0.1
FeM-UM	-18.36	0.27	-127.83	<0.01
DeM-UM	-33.05	0.01	-150.15	<0.001
FeM-UF	0.88	0.99	-47.68	0.05
DeM-UF	-13.81	0.44	-70	<0.01
DeM-FeM	-14.69	0.37	-22.32	0.60

654 Tukey HSD *post–hoc* pairwise comparison among treatments for guppies in Experiment 2. UM:
655 untreated males, UF: untreated females, DeM: males under demasculinisation, and FeM: males
656 under feminisation. A negative difference indicates that the second group in a treatment pair had
657 a higher parasite load than the first treatment.

658

659

660 **Table 4:** Guppy survival by treatment post-infection with *Gyrodactylus turnbulli.*

Coefficient	Estimate	SEM	Z-value	P (>|z|)
Untreated females	-2	0.47	-4.3	<0.001
Feminisation males	-1.16	0.37	-3.1	0.002
Demasculinisation males	-1.48	0.4	-3.67	<0.001

661 Cox proportional hazards results for survival until Day 13 after infection, "day of mortality" as a
662 response variable, and "treatment" as explanatory variable. Values are for individuals of a given
663 treatment compared to the untreated males.

664

665

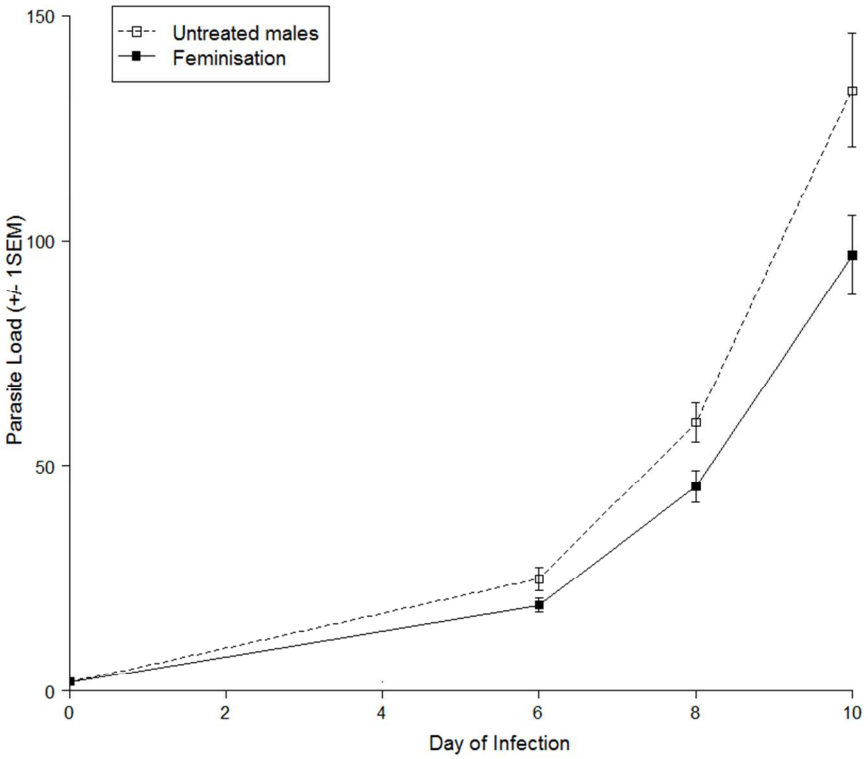

Fig 1: Mean Gyrodactylus turnbulli parasite load per host for untreated male guppies (dashed line) or males treated with flutamide and 17β-estradiaol (feminisation - solid line) by day of infection (Experiment 1). 332x285mm (72 x 72 DPI)

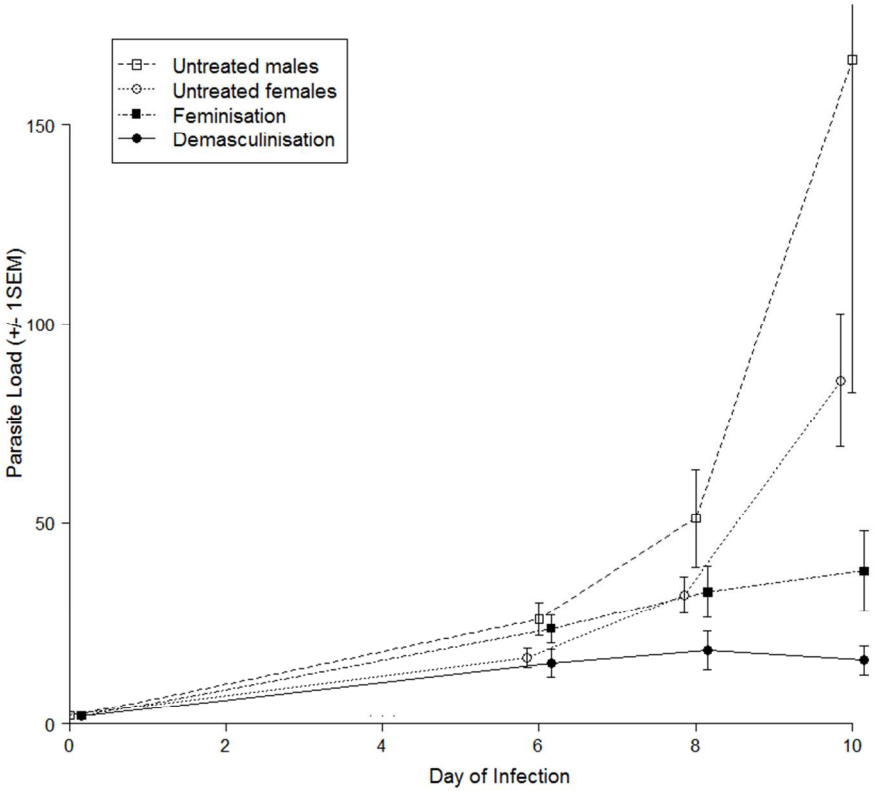

Fig 2: Mean Gyrodactylus turnbulli load per host in male guppies treated with flutamide (demasculinisation), with flutamide and 17β-estradiol (feminisation), and in untreated males and females, compared across days after infection (Experiment 2). Points are slightly offset on the x axis to reduce overlap.
332x285mm (72 x 72 DPI)

Supplementary Material

for

Demasculinisation of male guppiesincreases resistance to a common and harmful ectoparasite

Felipe Dargent, Adam R. Reddon, William T. Swaney, Gregor F. Fussmann, Simon M. Reader, Marilyn E. Scott, Mark R. Forbes

Table S1: Mean guppy standard length (SL) by treatment

	Treatment	Initial SL (mm ± s.e.m.)	Infection SL (mm ± s.e.m.)
Exp. 1	Untreated males	15.75 (±0.19)	15.60 (±0.2)
	Feminisation males	15.47 (±0.23)	15.49 (±0.2)
Exp. 2	Untreatedmales	15.36 (±0.26)	15.44 (±0.23)
	Untreatedfemales	17.97 (±0.3)	18.57 (±0.28)
	Demasculinisation males	15.79 (±0.29)	15.80 (±0.23)
	Feminisation males	15.24 (±0.27)	15.44 (±0.26)

Table S2: Mean guppy mass by treatment

	Treatment	Initial mass (g ± s.e.m.)	Infection mass (g ± s.e.m.)
Exp. 1	Untreated males	0.077 (±0.004)	0.074 (±0.003)
	Feminisation males	0.074 (±0.003)	0.075 (±0.003)
Exp. 2	Untreatedmales	0.085 (±0.004)	0.082 (±0.003)
	Untreatedfemales	0.127 (±0.006)	0.136 (±0.006)
	Demasculinisation males	0.091 (±0.006)	0.085 (±0.005)
	Feminisation males	0.084 (±0.006)	0.081 (±0.005)

15 **Table S3:** Sample size by population, treatment and day post-infection (Experiment 1).

Treatment	Day 0	Day 6	Day 8	Day 10
Untreated males	15	15	14	12
Feminisation males	14	14	14	14

16

17

Table S4: Sample size by treatment and day post-infection (Experiment 2).

Treatment	Day 0	Day 6	Day 8	Day 10
Untreatedmales	17	17	11	2
Untreatedfemales	19	19	17	13
Demasculinisation males	18	18	16	13
Feminisation males	18	18	18	12

Figure legends

Figure S5: *Gyrodactylus turnbulli* population growth trajectories on individual *Poecilia reticulata* hosts by hormone treatment (Experiment 1). Each line represents a separate individual.

Figure S6: *Gyrodactylus turnbulli* population growth trajectories on individual *Poecilia reticulata* hosts by hormone treatment (Experiment 2): A) Untreated control males, B) untreated control females, C) males under feminisation, and D) males under demasculinisation. Each line represents a separate individual.

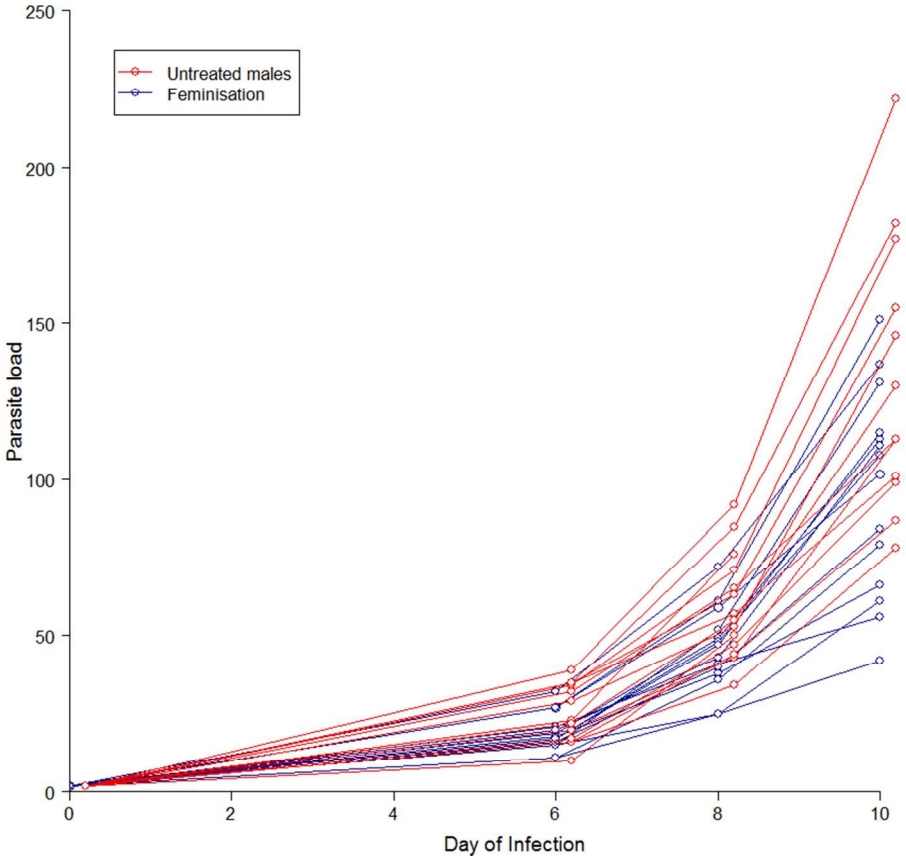

Fig S5: Gyrodactylus turnbulli population growth trajectories on individual Poecilia reticulata hosts by hormone treatment (Experiment 1). Each line represents a separate individual.
332x285mm (72 x 72 DPI)

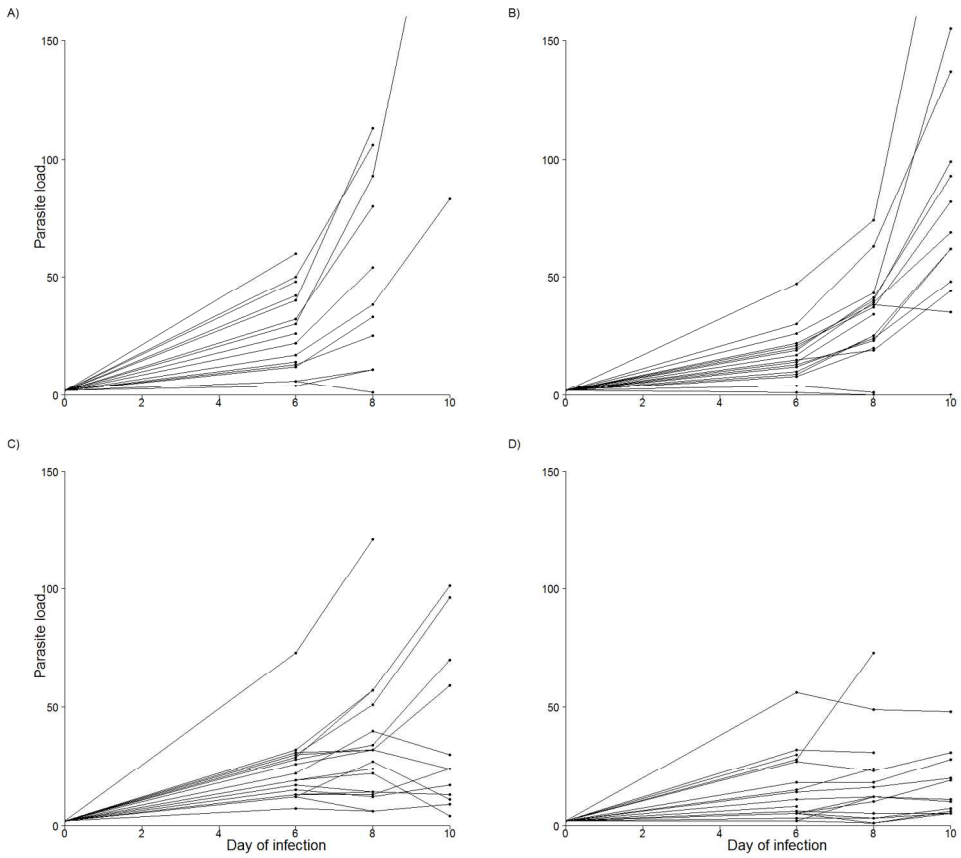

Fig S6: Gyrodactylus turnbulli population growth trajectories on individual Poecilia reticulata hosts by hormone treatment (Experiment 2): A) Untreated control males, B) untreated control females, C) males under feminisation, and D) males under demasculinisation. Each line represents a separate individual. 664x571mm (72 x 72 DPI)

Response to referees

We thank the referees for their constructive comments, which have strengthened the manuscript. We address each comment below and refer to the changes we made in the revised manuscript. The referee's original comments are in italics and our responses in plain font.

Referee 1

This experiment, reporting the impact of G. turnbulli on male guppies which had been chemically feminised, is long overdue; I am surprised it has not been undertaken before, and I was pleased to see it being done now. I started thinking the paper could be published more or less as it is, but more careful reading made me more cautious – certainly it needs more experimental detail, and if the authors on reflection can answer my concerns about the growth curve of the parasite population on the control fish, then it can be published; otherwise more work will be necessary. The MS is also overwritten in places and could do with some reduction.

We thank the referee for this strong endorsement that this is an important experiment. We have added the requested experimental details, and address the concern regarding the growth curve (see below). We have also edited the manuscript for length and made other changes to improve clarity.

1). I can find no mention of basic culture conditions for guppies prior to and during experimentation and chemical feminisation. No information on water type (tap, dechlorinated, artificial river water, salt supplementation, etc..), temperature of maintenance and experimentation,photoperiod or feeding regime, all of which can affect fish condition and hence performance of gyrodactylids, are given. It is mentioned (line 176) that water quality was monitored, but we haveno idea which parameters were measured, or how. Make the point early in the general methods that the fish used in the two experiments were of different stocks and came from different sources.

We now provide details on water characteristics before and during the experiment, and the temperature and photoperiod at which the laboratory set up was maintained (lines 130-140). These conditions were identical during the two experiments, and are very similar to the conditions used in previous work done at our laboratory (cited in manuscript as: Dargent, 2015; Dargent *et al.*, 2013). We have clarified how we monitored water quality: assessing clarity and accumulation of residues, as well as random sampling of chambers to test for alkalinity, pH, nitrite, nitrate, hardness and ammonia (lines 179-181). We now indicate early in the methods (lines 117-124) that we used different wild-derived guppy populations for the two experiments.

A key experimental factor, which is glossed over, is that the water was 75% changed every 4 days, and completely after 12 days, and we receive no details about the change rate during the experimental period. For me, this is a very long time to leave fish in the same water, and for example the Cable group change water every 2 days in a slightly smaller container. In my experience, water quality problems are the largest difficulty in getting consistent growth of gyrodactylid infections, although normally it works the other way to that seen here – it stops the parasites growing properly, rather than leading to near exponential growth! These points must be attended to before publication.

We were not clear about this point in the original manuscript. We now clarify that water changes were continued throughout the 31 days of experiment (i.e. the 21 days when fish received the hormone treatments and on the 10 subsequent days were the fish received the hormone treatments and were infected with *Gyrodactylus turnbulli*) (line 173). Thus the water was changed during the experimental period.

We agree with the reviewer's sentiment that keeping the fish in the same water for a long period of time could potentially be problematic. However, there are several reasons why we do not think that our approach to water quality affected the performance of the fish or the parasite in our experiment. First, the amount of food degradation in the tank was negligible given that we did not feed the fish *ad libitum* after the tanks were disconnected from the re-circulating system, but instead we fed them precise amounts of paste that were fully ingested (lines 159-161). Second, 1.8 litres is a relatively large volume of water for an individual that weighs an average of 0.08 grams. Third, parasite population growth in fish from the Lower Lalaja population

(Experiment 1) are in line with previous loads reported in our laboratory using a variety of guppy populations and the same parasite strain (Dargent, 2015; Dargent *et al.*, 2013). These earlier experiments were performed with the chambers attached to the recirculating systems (i.e. with complete renewal of water approximately every 8 minutes). Therefore it seems unlikely that procedures used for manual water changes had any significant impact on the parasite growth. We clarify these details in the manuscript (lines 179-183).

2). The normal pattern of G. turnbulli growth following inoculation with two parasites is of initial growth followed by decline, the turning point depending on the particular combination of host stock and parasite strain. Clearly the outcomes of the two experiments differ in this respect, possibly because they use different fish stocks, although differences in environmental conditions (potentially unsuspected or uncharacterised) could also cause this difference. These differences do not jeopardise the results because the two experiments can be regarded independently of each other; but some sort of explanation of the difference is needed. As I look at the results in Fig 1, given the tight error bars on these data, I would suspect that this fish stock/experiment did not show any decline in infection, but most fish sustained continuous parasite population growth (both treated and untreated). Experiment 2, with much bigger error bars towards the end of the experiment, and the different outcome of drug treatment, suggests to me that these fish limited parasite population growth much more effectively than the stock used in Experiment 1. I have to say, if my interpretation is right, I have never seen a stock as susceptible as that used in Experiment 1, especially to a pet shop strain of G. turnbulli, which are normally a bit pathetic compared to wild strains. Just crudely interpreted from the figure, the parasite on this stock regularly made 120 parasites from 2 in just 10 days; Cable and Oosterhout (2007 IJP 37, 1449-1458) peaked at a mean of around 40 parasites per fish, with the same starting conditions, using avirulent parasite strain from the wild. The tame Gt3 strain in the same experiments made only around 20 per fish. So there is something special about this combination of parasite and fish used in experiment 1.

As the reviewer notes, there are differences in the average parasite growth rate between the two experiments, our strain of *G. turnbulli* seems to have a higher growth rate on guppies descended from the Lower Lalaja population than on guppies descended from the Aripo by Quare mixed

population. That being said, we do not think it is plausible that these differences are caused by environmental (within the lab) differences, since both experiments were performed in the same location and following the same protocol, furthermore, the same parasite strain was used in both cases. It seems to us that the differences in *Gyrodactylus* dynamics are therefore most likely caused by differences between the host fish populations. As the reviewer suggests we now state in the manuscript that we consider interpopulation differences in resistance to be the most likely explanation for differences in parasite loads between the two experiments (lines 353-355).

The reviewer is correct in suggesting that the fish in Experiment 1 (Lower Lalaja population) did not reach the decline phase, here the difference between control and feminised males is due to differences in the rate of growth of the parasite (new Figure S5). We can confirm that the infections in Experiment 2 also did not reach a decline phase (new Figure S6), instead they seem to have had a slower rate of increase (with some individual exceptions). We interpret the larger error bars in Experiment 2 as a result of the high mortality of untreated control male subjects (truncated lines in new Figure S6 A). We speculate that control males in Experiment 2 would have had higher parasite loads and tighter error bars, perhaps similar to those of Experiment 1 control males, if not for the observed level of mortality. Yet, we agree with the reviewer in that the fish from Experiment 2 seemed more efficient at reducing the rate of growth of infection, particularly based on the trajectories of feminised males in both experiments. We have added the individual fish trajectories as a supplemental material (new Figures S5 and S6) to clarify the source of variation in the error bars between experiments (i.e. not due to a decline phase of infection) as well as to provide further detail on the infection dynamics.

The reviewer also mentions that the parasite loads reported in our fish are higher than those reported by other groups. However, this pattern is not unique to this study but consistent with our previous work (see above comment about water quality). We also cannot imagine environmental differences as an explanation for variation in parasite loads between Experiment 1 and Experiment 2. We now state in the manuscript that we did not detect signs of water degradation or of negative effects of water quality on the host or parasites (lines 179-183).

Parasite loads well over 40 *Gyrodactylus* at peak burden could have occurred because we only used fish that were naïve to *Gyrodactylus* (i.e. have never been infected or in direct contact with infected individuals) and whose mothers were also uninfected (i.e. removing possible maternal effects). Experiments that report peak *Gyrodactylus* loads of about 40 parasites may

have used fish that have been infected in the past and thus retained some degree of acquired resistance. For example, Cable and Van Oosterhout (2007) reported that strains of wild guppies retained acquired resistance at least for 53 days after they had cleared an infection (the longest period of time they tested), and they speculated that unlike domestic guppies (e.g. Scott, 1985), wild guppies might not lose their acquired resistance. Therefore, the low loads reported in experiments elsewhere could have been caused by individuals having some degree of acquired resistance. Since the above arguments are speculative, we would rather leave them out of the manuscript. Regardless of the cause of the relatively high *Gyrodactylus turnbulli* loads in our experiment, the key conclusions are not dependent on this observation, i.e. control fish had higher *G. turnbulli* loads than both feminised and demasculinised fish, independently of the differences in loads between the two experimental populations.

We think it is most likely that the difference in *Gyrodactylus* performance between experiments was caused by differences in the host populations used. Both experiments were performed using the same laboratory, machine, chamber model, water preparation methods, food type, food preparation methods and food delivery methods (lines 170-172). Thus, the use of different guppy populations seems the most parsimonious explanation for the difference in *Gyrodactylus* loads between the two experiments. Finally, although we can only speculate because of the high mortality in Experiment 2 (Figure S6), we argue that untreated control male loads were not very different between the two experiments. Indeed, on day 8, when there is still a large number of surviving control males in Experiment 2, control male parasite loads are very similar between the two experiments (Figures S5 and S6). In fact, what seems to have changed more strongly between the two experiments is the load on feminized males, which have a lower load on Day 10 for Experiment 2 than they do for Experiment 1. Our interpretation of this result would not be that the parasite – host-strain interaction is different between the two experiments; but that the guppies used in Experiment 2 are more responsive to the combined effect of flutamide and 17β-estradiol (feminisation), perhaps because (as suggested by reviewer 2) they trade-off more heavily between their investment in reproduction related traits and defence (i.e. they experience a higher cost of defence).

3). I think it would be very useful for the reader to see some individual trajectories for infections on individual guppies, because I am inferring this from means and error bars, which is not an ideal position to be in. It is perhaps worth mentioning that Ramirez et al. (2012, IJP 44, 809-817) roundly criticize the use of maximum likelihood statistical analyses of gyrodactylid population dynamics, because of the autocorrelative nature of gyrodactylid population growth which meansthat a bad performance in the first day or two of the infection (when effects are largely stochastic) can have a massive impact on population size later in the infection. This same group have a paper in press (or just out) with Parasites and Vectors detailing a Bayesian method (implemented in the freeware WinBugs) which allows you to estimate individual parasite population growth rates on a fish by fish basis, and then you can take a maximum likelihood approach to analyse the growth rates from each experimental treatment. I think this would greatly simplify the analysis presented here, and is not very intensive in terms of time needed. I am sure Raul Ramirez would supply thescripts if you wanted to try this method out.

As recommended by the reviewer we have plotted the individual trajectories for all guppies (new Figures S5and S6), by treatment, and added them to the supplementary materials. In this way we make the information easily available to interested readers.

We thank the reviewer for pointing us to the Ramirez et al. 2012 paper and subsequent work. We have included a statement in the manuscript referring to the autocorrelative nature of *Gyrodactylus spp.* population growth and its possible effect on interpretation of our results (lines 296-300). Although autocorrelation would seem to necessitate a repeated measures design, the mortality of individuals leads to an unbalanced design with respect to numbers of individuals assessed at later days post infection. Therefore, we were only able to use a repeated measures model for Experiment 1 but not for Experiment 2 because of the high mortality in the control group. The repeated measures model for Experiment 1 shows that *Gyrodactylus* loads on the untreated control males are significantly different from loads in feminised males, in agreement with the analysis on the original manuscript. We have also maintained the original analyses in the revised manuscript as they allow to distinguish at which days the differences in *Gyrodactylus* loads are significant.

We also contacted who we believe is the senior author of the Ramirez et al. 2015 unpublished/accepted manuscript. He kindly offered to send the manuscript if the first author agreed to make it available. Unfortunately, we were not able to get a copy of the manuscript. We look forward to exploring our past and present data in our future experiments, once the manuscript and scripts are published or available.

4). As it is, the difference between the experiments, which is probably due to the performance of the parasite on the two fish stocks, makes it impossible to draw conclusions which span the two experiments; one such is the highlight ′additional treatment with oestrogen (actually with asynthetic oestradiol, which is not the same, especially in a teleost such as the guppy) did not reduce parasitism further′. This conclusion is based on the two experiments – the first with both treatments simultaneously, the second with oestradiol and flutamide separated out. My interpretation of experiment 1, which gave such different results to experiment 2, is that this stock of fish was simply unable to respond to the parasite in the first place, and so treatment with the chemical feminizers did not make a great deal of difference. In experiment 2, a good response to infection is possible in this stock, which is inhibited by male sex hormones. So the feminising treatment had a much greater effect in experiment 2 because it unmasked a strong response to the parasites. It would be interesting to know the relative baseline titre of both and rogens and carotenoids in males of these two stocks of guppies.

Our explanation of Experiment 2 methods may have not been clear enough on how the feminisation treatment was performed. We have now clarified this point (line 224). In Experiment 2 we repeated the feminisation treatment used in Experiment 1 (i.e. guppies under feminisation received both flutamide and 17β-estradiol) and not, as the reviewer suggests, by only using 17β-estradiol. In Experiment 2 we had the additional treatment of giving only flutamide to a group of guppies (i.e. demasculinisation treatment). The inference that additional treatment with synthetic oestradiol (feminisation) did not reduce parasitism further is based on the comparison between the demasculinisation treatment and the feminisation treatment in Experiment 2. These two groups are not significantly different from each other but visual inspection (Figure 2 in the manuscript) suggests that demasculinised males had lower parasite

loads than feminised males (i.e. that the additional treatment with the synthetic oestradiol increased infection if anything), thus our statement is conservative.

We consider the use of different populations a strength of our experiment, because it allows us to make generalisations about the effects of androgens on defence against *Gyrodactylus turnbulli*. Indeed, as we mention in the results and discussion sections (lines 268-270, 290-293, 299-300, 355-358), in both experiments the treated populations had lower parasite loads than the untreated control population. The magnitude of this effect might well differ between populations for reasons alluded to by the reviewer.

We agree with the reviewer that, in hindsight, it would have been interesting to measure the relative baseline hormone and carotenoid concentrations of the guppies derived from the two different stocks. Yet we would not have been able to do so for the current experiments unless we had used different individuals. To our knowledge the methods for directly measuring carotenoid concentrations in male guppies involve killing the fish (e.g. Kolluru *et al.*, 2006) and given that *Gyrodactylus* infection affects carotenoid concentrations (e.g. Houde & Torio, 1992), this would have precluded our ability to test carotenoid concentration before infection and then infection dynamics on the same fish. Furthermore, given the size of guppies it is not possible to draw blood (i.e. to measure androgens) from an individual without killing the fish; while the alternative use of waterborne methods would have required a larger group of collaborators and would have considerably increased the cost of the study.

Minor points

5). There is a good chunk in the introduction detailing the difference in sex steroids between the sexes (!); not only is a lot of this literature rather old (to say the least), I thought the fact that males had more androgens than females was moderately well established by now. Cut this part of the introduction sharply.

As suggested by the reviewer, we have reduced most of this section. We clarify that the emphasis is not on the difference in sex steroids between the sexes but on the fact that hormones might drive sex differences in defence in two distinct ways: through their long-term effect on anatomical, physiological or behavioural differences associated with the development of each

sex and/or through current (shorter-term) effects in adulthood caused by differences in circulating levels of gonadal steroids.

6). The section on study system is also irrelevant, and the charms of gyrodactylids for experimental epidemiology are well known now. Delete, and any important parts can be placed straight into discussion or introduction as appropriate. Interestingly, there are several lines in the study system section (lines 119-123) dealing with carotenoids and parasite resistance which is much more modern and relevant than the points about brown trout and ketotestosterone in the introduction, but it misses the also highly relevant link between androgens, carotenoids and the immune system (e.g. McGraw & Ardia, 2007, Biology Letters 3, 375). I would have thought this a much more fruitful line to pursue in this MS.

We have reduced the information contained in the "The study system" section by about 30% but retained information that would be needed by the non-specialist reader to understand the relevant characteristics of both guppies and *Gyrodactylus*. We have also re-located to this section the information relative to the guppy's population of origin to, as suggested by the reviewer's first comment, make the point early that the fish used in the two experiments came from different origins (lines 117-124).

We thank the reviewer for suggesting the McGraw and Ardia (2007) paper. We now cite it to improve the discussion about the interactions between androgens and carotenoids (lines 372-377).

7). I am not sure what the tables add to the figures – I would tend to put the F statistics in the text and/or in the figure legends.

We would prefer to keep the tables and figures in the manuscript for two reasons. First, including more information in the figures would make them harder to read, and including this information in the text would also make it cumbersome. Second, our experience is that such tables facilitate extracting data for metaanalyses.

8). Typo in the legend for Fig 1 - estradial.

Thank you, fixed.

Referee: 2

Comments to the Author

The present study illustrates interactions of parasite infection rates (Gyrodactylus turnbulli) and gonadal steroids in guppies (Poecilia reticulate). Male guppies that had been demasculinised or feminised by food supplementation with an androgen receptor antagonist and/or oestrogen, showed lower infection rates with the parasite compared to sham treated males and females. This finding is interesting and may help a better understanding of variations in parasite burden in natural fish populations. The manuscript is well written and the experiments appear to be well conducted.

We thank the reviewer for these kind comments.

However, the conclusions made by the authors that androgens reduce the ability of guppies to resist infection (line 34) and are immunosuppressive (line 288), to my opinion can not be made based on the data presented here. Additional information on the immune status of the fish is needed to substantiate these assumptions. Accordingly, these statements should be reworded and formulated and discussed more carefully as a possible explanations for the observed phenomena. To my opinion, the observed reduction in parasite load might have been due to indirect effects, since demasculinisation and feminisation might have resulted in reduced investment in sexual traits and thereby indirectly have facilitated a stronger investment in immunity against the parasite.

As the reviewer suggests, increased resistance to *Gyrodactylus* could be caused by direct interference of androgens with the immune system or alternatively through allocation trade-offs related to investment in sex and immunity. We agree that the word “direct” (as mentioned in the last comment below) is thus misleading, nonetheless, in both alternative mechanisms reduced androgens levels lead to increased resistance (i.e. the host ability to control parasite burden) to the parasite. We have reworded the manuscript to avoid emphasis on the immunosuppressive

alternative and have stated in the conclusion section that further work is required to distinguish between these two viable options (lines 379-382).

With respect to the statistics used here, I wonder why 'infection time' was not included as a factor in the GLMs? Instead the authors seem to have calculated GLMs for each infection time point. Not sure if this was appropriate without correction for multiple testing. I would suggest to recalculate the GLMs with 'infection time' included as a factor.

Ideally, we would have been able to do a repeated measures GLM as suggested by the reviewer, yet the fact that so many individuals died before day 10 in the untreated control group precludes us from pursuing a balanced statistical test of any experimental effect, controlling for days post infection, in Experiment 2. We alert the reader to this constraint imposed by the parasite-associated mortality we observed (lines 205-207). Given that mortality was low in Experiment 1 we performed a repeated measures GLM (with negative binomial distribution) and found that, in accordance to our previous analysis, untreated control males had significantly higher *Gyrodactylus turnbulli* loads than feminised males (effect of treatment: $F_{1,77} = 4.94$, $p < 0.029$). We have made reference to this in the text (lines 256-257).. We have also maintained the original analyses in the revised manuscript to demonstrate when *Gyrodactylus* loads differ.

Minor comments:
I found it hard to understand the timelines of the two experiments; were the femininisation/demasculinisation periods the same for both experiments and were those continued after the parasite exposure? Please explain more explicitly in the materials and methods.

All treatments had the same duration in both experiments. Treatments with gonadal steroids started three weeks prior to infection with *Gyrodactylus turnbulli* and were maintained for the ten-day infection period. Treatments were stopped after the tenth day of infection in both experiments, but in Experiment 2 we continued to monitor survival for three more days (the

point at which all remaining fish were euthanized). We have now clarified this in the methods section (lines 166-168, 170-172, 249-250).

24 gonadal steroids?

Corrected. It now says "gonadal steroids".

47 This was not tested here and consequently was not a finding of the present study.

We have removed this finding from the list.

51 In doing so, ...

Corrected, thanks.

66-67 ...females in six of nine....of dessert rodents

We have added the word "out" to the sentence: "…in six [out] of nine…"

90 suggest to word this more cautiosly: ...steroids are ...'directly influencing the response to parasites', might rather be indirect.

We removed this sentence from the manuscript. Nonetheless, we follow the reviewer's advice and have changed the wording of other sentences to avoid confusing the reader. We now use "current" or "immediate" instead of "direct" to reflect the more immediate effects of circulating gonadal steroids (e.g. lines 78, 281). This current (short-term) effect is to be considered in contrast to the longer-term effects of sex hormone levels during development, which can have a lasting influence on individual physiology, anatomy and behaviour, and also influence resistance or defence and/or exposure of hosts to parasites.

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Title

Demasculinisation of male guppies increases resistance to a common and harmful ectoparasite

Authors

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Running title

Demasculinisation and ectoparasite resistance

SUMMARY

Parasites are detrimental to host fitness and therefore should strongly select for host defence mechanisms. Yet, hosts vary considerably in their observed parasite loads. One notable source of inter-individual variation in parasitism is host sex. Such variation could be caused by the immunomodulatory effects of gonadals steroids. Here we assess the influence of gonadal steroids on the ability of guppies (*Poecilia reticulata*) to defend themselves against a common and deleterious parasite (*Gyrodactylus turnbulli*). Adult male guppies underwent 31 days of artificial demasculinisation with the androgen receptor-antagonist flutamide, or feminisation with a combination of flutamide and the synthetic oestrogen 17β-estradiol, and their parasite loads were compared over time to untreated males and females. Both demasculinised and feminised male guppies had lower *G. turnbulli* loads than the untreated males and females, but this effect appeared to be mainly the result of demasculinisation, with feminisation having no additional measurable effect. Furthermore, demasculinised males, feminised males and untreated females all suffered lower *Gyrodactylus*-induced mortality than untreated males. Together, these results suggest that androgens reduce the ability of guppies to control parasite loads, and modulate resistance to and survival from infection. We discuss the relevance of these findings for understanding constraints on the evolution of resistance in guppies and other vertebrates.

Key words

gonadal steroids, feminisation, sex-biased parasitism, testosterone, fish, *Gyrodactylus, Poecilia reticulata*

Key findings

- ~~Blocking~~ Blockage of androgen receptors le~~a~~d to lower ectoparasite loads in male guppies
- Additional treatment with oestrogen did not reduce parasitism further
- ~~Untreated~~ Treated males experienced ~~*higher*~~*lower* parasite-induced mortality than untreated males
- ~~*Gyrodactylus* infections may mediate the effects of androgens on sexual selection~~

INTRODUCTION

Parasites are pervasive and are known to negatively influence host fitness by reducing reproductive output, growth rate, mating success, and survivorship (Price, 1980). In ~~so~~ doing so, parasites can ~~also~~ be influential drivers of ecological processes and evolutionary patterns (Hamilton, 1982; Hamilton & Zuk, 1982; Lafferty *et al.*, 2008; Minchella & Scott, 1991). Parasitism is expected to be a strong source of selection for defensive adaptations that allow hosts to control parasite numbers and mitigate parasite costs. When parasites are present, investment in costly defence mechanisms is expected to be favoured (Schmid-Hempel, 2011). Intriguingly, there is considerable within-population variation amongst individuals ~~within populations~~ in their susceptibility to parasites, suggesting that antiparasite defences are costly and/or trade-off with other fitness enhancing traits, and therefore that maximal defence may not be obtainable or adaptive for all individuals (Lazzaro & Little, 2009; Sheldon & Verhulst, 1996). A striking example of among-individual variation in parasite susceptibility is the common phenomenon of sex-biased parasitism, in which one sex is more frequently infected or carries larger mean parasite loads than the other (Forbes, 2007; Krasnov *et al.*, 2012; Nunn *et al.*, 2009; Zuk & McKean, 1996). For example Amo *et al.* (2005) found that wild male wall lizards (*Podarcis murallis*) had higher haemogregarine and ectoparasitic mite infection intensities than did females. Similarly, Krasnov *et al.* (2005) found higher flea abundance in males than females of six out of nine species of desert rodent.

Males and females differ in many ways that ~~and each of these differences~~ may partially account for sex differences in parasite infection rates. For example, males and females often differ in body size and larger individuals typically have more parasites (Guégan *et al.*, 1992; Poulin & Rohde, 1997). Males and females may also be exposed to parasites at different rates

due to sex differences in space use or social behaviour (Tinsley, 1989). Furthermore, sex differences in time and energy allocation to sexual activities (e.g. courting and fighting) and resource acquisition also could drive sex differences in parasite loads through differences in the amount of resources available for investment in defence (Zuk, 1990).

Gonadal steroids play a critical role in sexual differentiation during development, resulting in sex differences in anatomy, physiology, and behaviour (Arnold, 2009; Wallen & Baum, 2002), and therefore may have a long-term~~indirectly~~ influence on sex-biased parasitism ~~through~~ by organizing phenotypic characteristics during development which in turn affect parasite defence later in life~~development~~. However, gonadal steroids also can have a more immediate influence on sex-biased parasitism because~~as~~ variation in circulating hormones~~gonadal steroids~~ in adults can ~~also~~ mediate sex differences in immune function (Grossman, 1989; Zuk & McKean, 1996)~~, as the levels of and response to these hormones often differ dramatically between males and females (Feder, 1985). For example male brown trout (*Salmo trutta*) have higher circulating levels of the primary teleost androgen 11-ketotestosterone than females (Kime & Manning, 1982) and concordantly, males from both wild and hatchery populations have higher prevalence and more severe infections of the ectoparasites *Gyrodactylus* spp., *Ichthyophthirius* spp. and *Scyphidia* spp. than females (Pickering & Christie, 1980). Furthermore, male brown trout also show decreased parasite resistance when dosed with exogenous testosterone (Buchmann, 1997). Therefore, gonadal steroid hormones may play a dual role in determining parasite resistance in adult animals by both organizing phenotypic characteristics during development which in turn affect parasite defence later in life, and by directly influencing the response to parasites in adult animals.~~ Understanding precisely how circulating gonadal steroids influence defence is a crucial step in understanding individual

variation in defence, as well as the potential for and magnitude of sex-biased parasitism, which in turn are ~~essential~~ necessary for understanding host-parasite dynamics in natural systems. To this end, it is essential to evaluate both the role of gonadal steroids during development and the role that circulating gonadal steroids play in parasite resistance in adults.

~~In the current study,~~Here, we ~~use~~ studied guppies (*Poecilia reticulata*) derived from wild populations and their common and harmful ectoparasites (*Gyrodactylus turnbulli*) to address the importance of ~~this second role of~~ circulating gonadal steroids in determining antiparasite defences, i.e. the effect that steroid hormone systems have on adult resistance to parasites. To this end we manipulated gonadal steroid levels in adult guppies by administering an androgen receptor antagonist (to demasculinise them), or a combination of an androgen receptor antagonist and an artificial oestrogen (to demasculinise and then feminise them), before assessing their resistance to *G. turnbulli*.

MATERIALS AND METHODS

The study system

The guppy is a sexually-dimorphic poeciliid fish native to the island of Trinidad and Northern South America (Magurran, 2005). *Gyrodactylus turnbulli* is a highly prevalent (Harris & Lyles, 1992) and deleterious ectoparasite of wild guppies (Gotanda *et al.*, 2013; van Oosterhout *et al.*, 2007a). These monogenean flatworms transmit through host-to-host contact, and attach to their host's epithelium where they feed and give birth to flukes with fully developed embryos "in-utero" (Bakke *et al.*, 2007). Therefore, *Gyrodactylus* infections are prone to exponential population increase on individual hosts and epidemic dynamics within guppy

populations (Scott & Anderson, 1984), which leads to high guppy mortality in the laboratory (Dargent *et al.*, 2013a; Van Oosterhout *et al.*, 2007b) and the wild (van Oosterhout *et al.*, 2007a).

The guppy-*Gyrodactylus* host-parasite system is a convenient model to assess the role of gonadal steroids in the defence against parasites. First, the regulatory effect of androgens on guppy behaviour and colouration may play a critical role in the expression of secondary sexual characters and mating success (Bayley *et al.*, 2002; 2003). Second, correlations between carotenoid colouration, mate preference and defence against parasites have long been recognised in guppies (Houde & Torio, 1992; Kennedy *et al.*, 1987; Kolluru *et al.*, 2006) while the ecological and evolutionary drivers of guppy parasite defence have been the focus of much recent research (Dargent *et al.*, 2013a; Dargent *et al.*, 2013b; Fitzpatrick *et al.*, 2014; Gotanda *et al.*, 2013; Perez-Jvostov *et al.*, 2012; Pérez-Jvostov *et al.*, 2015; Tadiri *et al.*, 2013). Missing from this increasingly well-understood model system is the degree to which circulating gonadal steroids influence defence against *Gyrodactylus* parasites in the guppy. ~~Field evidence suggests that sex hormones could play an important role in regulating guppy defence against *Gyrodactylus*. Guppies show sex-biased *Gyrodactylus* parasitism in the wild, with females carrying higher *Gyrodactylus* loads than males at sites where predation is high and the reverse pattern at sites where predation is low (Gotanda *et al.*, 2013). Additionally, common garden laboratory experiments on isolated guppies report sex-biased parasite loads despite controlling for many of the ecological and behavioural factors commonly assumed to underlie sex differences in *Gyrodactylus* loads .~~

Guppies used in this research were laboratory-reared from fish collected in Trinidad. In Experiment 1, we used F2 descendants from a Trinidadian population collected in 2013 after having been experimentally translocated in 2009 (Travis *et al.*, 2014) from a high-predation site

in the Guanapo river where *Gyrodactylus* spp. was present to a tributary stream (Lower Lalaja) where predation was low and *Gyrodactylus* was absent. In experiment 2, we used F1 descendants of wild-caught fish collected between 2010 and 2011 from the Aripo and Quare rivers from sites where predation is high and *Gyrodactylus* spp. is present. These guppies were kept together as a mixed origin population.

Hormone treatments

Two weeks prior to the start of the hormone treatments, sexually mature guppies were isolated into 1.8 L chambers in an aquatic housing system (Aquaneering Inc., San Diego, USA). The fish were physically isolated but retained visual contact with their neighbours throughout the experiments. ~~Subjects were fed *ad libitum* with TetraMin Tropical Flakes (Tetra, Melle, Germany) ground into powder and reconstituted with water to form a thick paste that was delivered using Hamilton microliter syringes (Hamilton Laboratory Products, Reno, USA).~~ The laboratory was maintained at 23 ±1°C with a 13 h 11 h (L:D) photoperiod. We used carbon-filtered municipal water that was conditioned with Prime (Seachem Laboratories , Madison, USA), Freshwater Biozyme (Mardel, Oklahoma city, USA), and left to stand for two days and warm up before being added to the housing systems. The housing system passed water through a filter pad, a biological filter, a set of carbon filters and a UV sterilization device. Subjects were fed with TetraMin Tropical Flakes (Tetra, Melle, Germany) ground into powder and reconstituted with water to form a thick paste that was delivered using Hamilton microliter syringes (Hamilton Laboratory Products, Reno, USA). Prior to the start of the hormone treatments subjects were fed *ad libitum* and their chambers remained connected to the re-

circulating system, thus each chamber had a complete water change ~~turnover~~ approximately every 8 minutes.

We gathered data on individual body size (measured as standard length; ~~–~~ SL) and mass at two time points: on the first day we began administering the hormone treatments, and 21 days later, the day we began experimental parasite infections (Tables S1, S2). To measure SL and mass we anesthetised each guppy in 0.02% Tricaine Methanesulfonate - MS222 (Argent Chemical Laboratories, Redmond, USA) buffered to a pH of 7 with $NaCO_3$. Guppies were then weighed to the nearest 0.01 g and photographed on the left side with a Nikon D90 camera (Nikon, Mississauga, Canada). Each image included a ruler for scale.

At the start of the hormone treatments, male guppies (mean mass = 0.08 g ±0.002 s.e.m.) were randomly assigned to ~~either~~ control, demasculinisation or feminisation treatments, while females (mean mass = 0.13 g ±0.006 s.e.m.) remained untreated. Acetone was used as a solvent to combine the pharmacological agents with ground flake food. We saturated the food with acetone mixed with the hormone treatment and then allowed the acetone to evaporate in a fume hood for 24 hours. Untreated control male and female guppies received food that had been saturated with acetone alone without any pharmacological treatment, guppies in the demasculinisation treatment received food that had been dosed with 4.29 mg of the androgen receptor antagonist flutamide (Sigma-Aldrich, Oakville, Canada) per gram of dry food, and guppies in the feminisation treatment received food that had been dosed with 4.29 mg of flutamide and 0.04 mg of the synthetic oestrogen 17β-estradiol (Sigma-Aldrich, Oakville, Canada) per gram of dry food. Each guppy received 5 µL/day of paste prepared with their respective treatments (in a 7:8 food:water ratio), which is equivalent to 10.40 µg/day/guppy of flutamide and 0.10 µg/day/guppy of 17β-estradiol. Guppies ingested all of the food provided to

them. The ~~dose of~~ flutamide~~/g body weight~~dosage was based on previous dose-response studies in guppies showing effective inhibition of male-specific traits (Bayley *et al.*, 2003; Kinnberg & Toft, 2003), without the increased mortality seen at higher doses (Baatrup & Junge, 2001). The dose of 17β-estradiol/g body weight was based on dose-response work in goldfish demonstrating robust inhibition of male-specific traits, but no associated weight loss (Bjerselius *et al.*, 2001). All hormone treatments lasted for 31 days (i.e. 21 days of treatment without parasite infections and 10 days of treatment after *Gyrodactylus* infection). We performed two consecutive experiments. Experiment 1 had two treatments: feminisation of males and untreated males. Experiment 2 had the same treatments as Experiment 1 in addition to demasculinisation of males and untreated females. These experiments were identical in all regards with the exception of the additional treatments (see below) and the use of different wild-derived guppy populations ~~(see below)~~.

During the 31 days of hormone treatment, the guppies remained in their 1.8 L chambers, which we disconnected from the aquatic ~~flow-through~~recirculating system, chemically isolating the fish to ensure that no hormone treatment passed between the chambers. Visual contact between neighbours was retained throughout the experiment and therefore the fish were not socially isolated at any time. To maintain water quality during the treatment period, we changed 75% of the water in each chamber every four days and replaced the chamber with an entirely fresh one every 12 days. Water quality was monitored throughout the experiments by performing visual checks for water clarity and residue presence and by weekly tests, in randomly selected chambers, of alkalinity, pH, nitrite, nitrate, hardness and ammonia ~~throughout the experiments and~~. ~~w~~Water quality was within normal range throughout and we did not detect any sign of water quality degradation at any time, or of negative effects of water quality on the hosts or parasites.

Experimental Infections

21 days after the start of the hormone treatments, all fish were individually anaesthetised in 0.02% MS222 and infected with two *Gyrodactylus turnbulli* each. We infected each guppy by removing a small piece of fin tissue or a scale carrying *G. turnbulli* from a euthanized infected donor guppy and placing it next to the recipient guppy's caudal fin until we saw, under a Nikon SMZ800 dissecting ~~micro~~stereoscope (Nikon Instruments, Melville, USA), that two *G. turnbulli* had attached to the experimental fish. After infection, each guppy was allowed to recover from anaesthesia in its home chamber. We monitored *G. turnbulli* numbers on each live subject on days 6, 8 and 10 post infection, by anaesthetising the fish and counting the parasites using the ~~a Nikon C-BD115~~ dissecting stereoscope ~~(Nikon Instruments, Melville, USA)~~ at 18x magnification. We used *G. turnbulli* from our laboratory population, which was initially obtained in 2009 from domestic guppies purchased from a commercial supplier in Montreal, QC, Canada. This *G. turnbulli* population has been maintained on domestic-origin host guppies, and therefore has not had any period of coevolution with the wild-origin guppy populations used in this study.

Analysis

To assess whether hormone treatment and guppy body size (SL) had an effect on *G. turnbulli* load on each count day, we fitted a generalised linear model (GLM) with a negative binomial distribution and a log link function using Tukey HSD for pairwise *post-hoc* comparisons. To assess the general effect of hormone treatment on the growth trajectory of *G. turnbulli* we fitted a repeated measures GLM with a negative binomial distribution for Experiment 1~~, but not for~~. We were unable to perform this analysis for Experiment 2 because of the high parasite-induced

mortality in the untreated control group. The repeated measures GLM was conducted in SPSS 22 (IBM, New York, USA), all remaining analyses were conducted using the R Language and Environment for Statistical Computing v 3.1.0 (R Development Core Team, 2014). α was set at $p<0.05$. Data are archived in the Dryad repository (link to be added).

Experiment 1

To assess whether guppy resistance was influenced by the action of circulating gonadal steroids we compared 15 untreated males to 14 males that had been treated simultaneously with flutamide (an androgen receptor antagonist) and 17 β-estradiol (a~~n~~ synthetic oestrogen) (Table S3). Guppy body size and mass did not significantly differ between treatments (feminisation vs. untreated) at the start of the experiment (SL: $F_{1,27}=0.91$, $p=0.35$; mass: $F_{1,27}=0.23$, $p=0.63$), nor at the start of infection (i.e., 21 days after the start of hormone treatment; SL: $F_{1,26}=0.14$, $p=0.71$; mass: $F_{1,27}=0.01$, $p=0.91$). Subjects were laboratory-reared F2 descendants from a Trinidadian population experimentally translocated in 2009 (Travis *et al.*, 2014). ~~The ancestral population was translocated from a high-predation site in the Guanapo river where *Gyrodactylus* spp. was present to a tributary stream (Lower Lalaja) where predation was low and *Gyrodactylus* was absent, and was collected from the latter location in 2013 (Travis *et al.*, 2014).~~

Experiment 2

To disentangle the relative roles of androgens and oestrogens, we ran a second experiment, repeating both treatments in Experiment 1 along with two additional treatments: male demasculinisation and untreated females, resulting in four total treatment groups (Table S4). Males under demasculinisation were treated with flutamide only, allowing us to investigate male

parasite resistance when androgen receptor signalling is blocked. Different gonadal steroids can have contrasting effects on immune function: androgens generally have immunosuppressive effects, while oestrogens often promote disease resistance, although effects can vary (Klein, 2000; 2004). We also tested untreated females to check for sex-biased parasite resistance in untreated guppies, as this is known to vary between populations (Dargent, 2015; Gotanda *et al.*, 2013; Stephenson *et al.*, 2015).

~~Subjects in Experiment 2 were laboratory-reared guppies derived from wild-caught fish collected between 2010 and 2011 from the Aripo and Quare rivers in Trinidad from sites where predation is high and *Gyrodactylus* spp. is present. These guppies were kept together as a mixed origin population.~~ As is typical for guppies, the females were ~~of~~ larger ~~SL~~ than the males, both at the beginning of the experiment (mean ± s.e.m. SL: males=15.46 ±0.16, females= 17.97 ±0.3; $F_{3,65}$=21.28 , p<0.001) and at the time of infection (mean ± s.e.m. SL: males=15.56 ±0.14, females=18.57 ±0.28; $F_{3,68}$=36.99, p<0.001). ~~However, t~~There was no significant ~~SL~~ difference in SL among the three male treatments at either time point (start of treatments: $F_{2,47}$=1.1, p=0.34; infection: $F_{2,50}$=0.72, p=0.49). A similar pattern was observed for body mass. Female guppies were heavier than males when they started receiving the hormone treatments (mean ± s.e.m.: males=0.09 ±0.003, females=0.13 ±0.006; ($F_{3,65}$=14.73, p<0.001) and on the first day of infection (mean ± s.e.m.: males=0.08 ±0.003, females=0.14 ±0.006; $F_{3,68}$=31.17, p<0.001), but, mass did not differ between male treatments at the start of the ~~treatment~~ experiment ($F_{2,47}$=0.38, p=0.68) nor on the day of infection ($F_{2,50}$=0.24, p=0.79). Males did not differ in SL between Experiment 1 and 2 (initial SL: $F_{1,77}$=0.42, p=0.52; infection day SL: $F_{1,79}$=0.004, p=0.95) but males in Experiment 1 were lighter than those in Experiment 2~~did differ in mass~~ (initial mass:

278 $F_{1,77}$=6.21, p=0.01; infection day mass: $F_{1,80}$=4.53, p=0.04; ~~),): males in Experiment 1 were~~
279 ~~slightly lighter (~~Tables S1, S2).

280 Post-infection mortality was high in Experiment 2 and so we used a Cox proportional
281 hazards model to determine whether hormone treatment and body size (SL) influenced guppy
282 survival up to 13 days post infection (i.e. three days after we had finished treating the guppies
283 with hormones~~the hormone treatments had finished~~). Standard length and its interaction with
284 hormone treatment had no significant effects on survival and thus were dropped from the model
285 by AIC step-wise model selection.

286

287 RESULTS

288 *Experiment 1*

289 Guppies that underwent feminisation via treatment with flutamide and 17β-estradiol had
290 significantly lower *G. turnbulli* loads than untreated guppies throughout the infection period
291 (repeated measures GLM effect of treatment: $F_{1,77} = 4.94$, $p < 0.029$), and specifically on both
292 Day 8 and Day 10 of infection (Table 1, Figure 1, Figure S5). We did not detect any significant
293 effect of SL or of its interaction with the hormone treatment on parasite load (Table 1). Mortality
294 following infection was low. Only 3 individuals had died (10%) by Day 10 of infection, all of
295 which were in the untreated group (Table S3). *G. turnbulli* populations on individual guppies
296 continued to grow through the duration of the experiment (Figure S5). We observed no obvious
297 pathological effects of treatment with flutamide and 17β-estradiol in concert (feminization), and
298 this treatment significantly increased resistance to *Gyrodactylus turnbulli* on~~in~~ all ~~male~~ guppies.

299

300 *Experiment 2*

Hormone treatment had a significant effect on parasite load at both Day 8 and Day 10 (Table 2, Figure 2, Figure S6). As in Experiment 1, males that underwent feminisation also had lower *G. turnbulli* loads on both Day 8 and Day 10 of infection compared to untreated males (Tables 2, 3, Figure 2), although this difference was only statistically significant on Day 10. Males that underwent the demasculisation treatment had significantly lower *G. turnbulli* loads compared to untreated males on Day 8 and 10 of infection (Tables 2, 3, Figure 2). ~~As in Experiment 1, males that underwent feminisation also had lower *G. turnbulli* loads on both Day 8 and Day 10 of infection compared to untreated males (Tables 2, 3, Figure 2), although this difference was only statistically significant on Day 10.~~ Parasite loads were not significantly different between those males that underwent demasculinisation and those that underwent feminisation at any time point, and both had lower loads than untreated females on Day 10 (Tables 2, 3, Figure 2). With few exceptions, *G. turnbulli* populations on individual guppies continued to grow for the duration of the experiment while their hosts remained alive, but growth trajectories differed with treatments (Figure S6). We observed no significant effects of SL or any interaction effects between body size and treatment on parasite load (Table 2). Contrary to previous studies on wild guppy populations (Gotanda *et al.*, 2013), we found no evidence that guppies from our Aripo/Quare mixed-origin laboratory-bred population were sexually dimorphic in *G. turnbulli* resistance (Table 3). In contrast to Experiment 1, guppy mortality after infection with *G. turnbulli* was high in the mixed Aripo/Quare population: 67% of all fish had died by the 13th day of infection (56% by the 10th day). This mortality was significantly higher in the untreated males than in either group of treated males (demasculinisation or feminisation) or the untreated females (Tables 4, S2).

DISCUSSION

We conducted two independent experiments with different populations of wild-origin guppies and found that ~~the action of~~ gonadal steroid~~s~~ affects the ability of male guppies to control infection by the ectoparasite *Gyrodactylus turnbulli*. *G. turnbulli* populations on individual hosts increased over the experiment, but t~~T~~reatment with the androgen receptor antagonist flutamide (resulting in 'demasculinised' males) or a combination of flutamide and the oestrogen 17β-estradiol (resulting in 'feminised' males) resulted in reduced *G. turnbulli* loads compared to untreated males or females. These differences were not explained by differences in body size. Furthermore, males under both feminisation and demasculinisation treatments showed significantly greater survival compared to untreated males following infection in our second experiment. Variation in *G. turnbulli* population growth within treatments and between experiments is likely to be influenced by the autocorrelative nature of *Gyrodactylus* population growth (Ramírez *et al.*, 2012), yet the effects of gonadal steroid manipulation generated significantly different parasite loads between treatments in both experiments. Taken as a whole, these results suggest that androgens have a~~n immunosuppressive~~ detrimental effect ~~in the~~on guppy resistance to parasitism.

To our knowledge, only one previous study has experimentally assessed the role of gonadal steroids on *Gyrodactylus* resistance. Buchmann (1997) evaluated the effect of testosterone on female trout (*Oncorhynchus mykiss*) resistance to *Gyrodactylus derjavini* and concluded that testosterone injections le~~a~~d to higher parasite loads. However, the results of the Buchmann (1997) study could not distinguish between a~~n~~ detrimental ~~immunosuppressive~~ effect of testosterone on ~~the~~ host defence and the alternative hypothesis that testosterone has a direct positive effect on *Gyrodactylus* reproduction. Our results suggest that a~~n immunosuppressive~~

detrimental effect of androgens on the host is more likely than a direct effect of testosterone on *Gyrodactylus* reproduction. Our experimental fish received flutamide, which binds to androgen receptors broadly inhibiting the host physiological response to multiple androgens in teleost fishes (including both testosterone and 11-ketotestosterone; de Waal *et al.*, 2008; Jolly *et al.*, 2006) without altering the circulating levels of these hormones (Jensen *et al.*, 2004).

Oestrogens also may have immunomodulatory effects in teleost fishes (e.g. Cuesta *et al.*, 2007; Watanuki *et al.*, 2002), but the degree to which they enhance or reduce host immunity seems to be highly system and species-specific (Chaves-Pozo *et al.*, 2012). When we consider the role of oestrogens on defence against *Gyrodactylus*, two lines of evidence suggest that it did not have a major effect in the guppy. First, male guppies treated with flutamide and 17β-estradiol did not differ in resistance from male guppies treated with flutamide alone, suggesting that 17β-estradiol did not have a substantial additional ~~immunomodulatory~~ effect on defence. Second, untreated female guppies were not more resistant than males that underwent demasculinisation and, in fact, they had higher parasite burdens on Day 10 of infection.

Female guppies are larger than males and sexual dimorphism in body size is a common explanation for sex-biased parasitism in vertebrates. The larger sex is expected to have higher parasite loads since larger individuals have a wider area exposed to parasite attacks or a larger resource base for the parasite population to grow (Zuk & McKean, 1996). However, we did not detect a difference in parasite loads between untreated males and females, nor did body size correlate with variation in resistance in either experiment. This finding might appear surprising, given that field surveys (Gotanda *et al.*, 2013) and laboratory experiments (Dargent, 2015) report sex differences in *Gyrodactylus* load in certain guppy populations, and in at least one instance such a sex difference has been linked to size dimorphism (Cable & van Oosterhout, 2007).

However, sex-biased parasitism in guppies is not consistently male biased and appears to be influenced by ~~interactions with~~ predation regime. For example, Gotanda *et al.* (2013) reported higher *Gyrodactylus* spp. loads on females ~~than on~~compared to males in natural streams where the risk of predation was high but the reverse pattern at sites where the risk of predation was low, suggesting that body size differences are not a comprehensive explanation for sex-biased parasite loads in guppies. Furthermore, experimentally translocated wild guppies have been shown to rapidly evolve resistance to *Gyrodactylus* in a sex-specific manner, leading to the loss of sexual dimorphism in resistance (Dargent, 2015). Thus, it is possible that the population we used for Experiment 2 is one that shows minimal sexual dimorphism in resistance to *Gyrodactylus*, although we did observe ~~considerably~~ higher mortality in untreated males than females. It is possible that the high mortality in the untreated male group, which considerably reduced our sample size, precluded our ability to detect an otherwise significant dimorphism in parasite loads. Nevertheless, our results clearly show that regardless of any initial sexual dimorphism in defence, interfering with androgen signalling augments resistance to *G. turnbulli* in male guppies.

The significantly higher mortality of untreated males compared to untreated females suggests that androgens may reduce guppy tolerance to *Gyrodactylus* (i.e. the ability of hosts to reduce the negative impacts of a given parasite load; Raberg et al. 2007). This line of reasoning is supported by the lower mortality of males that underwent both demasculinisation and feminisation compared to the untreated males. We did observe a difference in untreated male mortality between Experiments 1 and 2, possibly the result of population differences in subjects' susceptibility to *Gyrodactylus* induced mortality. Given that laboratory conditions were identical between the two experiments, the most likely cause for particular differences in mortality and

parasite loads between Experiment 1 and Experiment 2 is the fish population of origin. However, regardless of population origin, guppies that underwent hormone treatments (demasculinisation or feminisation) experienced lower mortality during infection and carried lower parasite loads than untreated males in both experiments.

The suppressive effect of the androgen system on guppy defence against the model monogenean *G. turnbulli* suggests a trade-off between resistance to these ectoparasites and other fitness-related traits of male guppies. On the one hand, female guppies typically prefer to mate with males that show brighter carotenoid colouration (Houde & Endler, 1990) and more active courtship (Kodric-Brown & Nicoletto, 2001), traits which have positive correlations with circulating androgens (Baatrup & Junge, 2001; Bayley *et al.*, 2003), and thus higher levels of circulating androgens would seem to increase male fitness. On the other hand, infections by *Gyrodactylus* are known to decrease male carotenoid colouration and display rate, and consequently decrease female preference for males with higher *Gyrodactylus* loads (Houde & Torio, 1992; Kennedy *et al.*, 1987). Furthermore, *Gyrodactylus* infection may compromise predator evasion, for example via increased morbidity and decreased swimming performance (Cable *et al.*, 2002). Thus *Gyrodactylus* can decrease male guppy host fitness through the direct effect of increased mortality and through the indirect effect of decreased mating opportunities, which may counterbalance the fitness enhancing properties of their androgen hormones. A further possibility is that increases in circulating androgens could promote carotenoid accumulation, that in turn counterbalances the immunosuppressive effects of androgens (e.g. Blas *et al.*, 2006; McGraw & Ardia, 2007). However, this possibility is unlikely here, given that males with intact androgen levels had higher parasite burdens than those ~~which had been~~ under the feminisation and demasculinisation treatments.

In conclusion, a reduced response of androgen receptors to circulating androgens was found to lead to decreased parasite burdens and parasite-induced mortality. Future work should determine if androgens have a direct immunosuppressive effect or, alternatively, if androgen dependent changes in sexual traits and reproductive investment indirectly affects investment in immunity. Our findings are consistent with the idea that androgens modulate immune function but run contrary to the view that size determines parasite loads, and therefore help further the understanding of inter-individual variation in parasitism. The developmental and ~~direct~~ current (circulating) effects of gonadal steroids on the immune system and resistance to infection, as well as their indirect effects on secondary sexual traits that affect fitness, are underappreciated~~often ignored~~ in studies addressing the ecology and evolution of vertebrate defence against parasites. Our results on a model host-parasite system strongly suggest that gonadal steroids should be considered in concert with morphological or behavioural differences when accounting for variation among individuals and between the sexes.

ACKNOWLEDGEMENTS

We thank HJ Pak, K White, D Uthayakumar and G Daggupati for laboratory assistance, A Morrill and S Portalier for figure coding advice, and A Hendry for use of his aquatic housing systems (NSERC-RTI #148297). We thank D Reznick, C Ghalambor, E Ruell, D Fraser, and the FIBR team for supplying us with guppies used in Experiment 1.

FINANCIAL SUPPORT

We thank the Quebec Centre for Biodiversity Science (FD); the Natural Sciences and Engineering Research Council of Canada (NSERC) (GFF - #356373-07; SMR - #418342-2012 and #429385-2012; ARR), Richard H. Tomlinson fund (ARR) and the Canada Foundation for Innovation (SMR - #29433). Research at the Ghalambor lab was supported by a NSF Faculty Early Career (DEB-0846175). The guppy introductions were funded by a United States NSF-Frontiers in Integrative Biological Research grant to D Reznick P.I. (EF-0623632).

ETHICAL STATEMENT

This study was carried out in accordance with the regulations of the McGill University Animal Care Committee (AUP #5759) and the guidelines of the Canadian Council on Animal Care.

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Legends to figures

Figure 1: Mean *Gyrodactylus turnbulli* parasite load per host for untreated male guppies (dashed line) or males treated with flutamide and 17β-estradiaol (feminisation - solid line) by day of infection (Experiment 1).

Figure 2: Mean *Gyrodactylus turnbulli* load per host in male guppies treated with flutamide (demasculinisation), with flutamide and 17β-estradiol (feminisation), and in untreated males and females, compared across days after infection (Experiment 2). Points are slightly offset on the x axis to reduce overlap.

Tables

Table 1: *Gyrodactylus turnbulli* parasite load is significantly higher in untreated male guppies compared to males under feminisation on Day 8 and 10 of infection (Experiment 1).

	Day 6[a]	Day 8[b]	Day 10[c]
Hormone treatment	3	5.49*	5.01*
SL	2.73	2.44	1.18
Treatment:SL	0.83	0.35	0.12

[a] n=29; [b] n=28; [c] n=26.

Generalized linear model results for *Gyrodactylus turnbulli* load (integer variable with a negative binomial distribution) on guppies for days 6, 8 and 10 of infection, with treatment (feminisation vs. untreated males) as factor and guppy standard length (SL) as a covariate. F-values reported, significant differences in bold (*=$p<0.05$).

680 **Table 2:** *Gyrodactylus turnbulli* parasite load varies with sex and hormone treatment on Day 8
681 and 10 of infection (Experiment 2).

	Day 6[a]	Day 8[b]	Day 10[c]
Hormone treatment	2.5	3.26*	11.25***
SL	0.81	0.26	0.29
Treatment:SL	0.49	0.24	2.68

682 [a] n=72; [b] n=62; [c] n=40.

683 Generalized linear model results for *Gyrodactylus turnbulli* load (integer variable with a negative
684 binomial distribution) on guppies for days 6, 8 and 10 of infection, with treatment (untreated
685 males, untreated females, males under demasculinisation, and males under feminisation) as
686 factor and guppy standard length (SL) as a covariate. F-values reported, significant variables in
687 bold (*=p<0.05, ~~**=p<0.01,~~ ***=p<0.001).
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689

690 **Table 3:** *Post-hoc* pairwise comparisons of *Gyrodactylus turnbulli* load by treatment

691 (Experiment 2)

Treatment pair	Day 8		Day 10	
	diff.	adj. p	diff.	adj. p
UF-UM	-19.25	0.24	-80.15	0.1
FeM-UM	-18.36	0.27	-127.83	<0.01
DeM-UM	-33.05	0.01	-150.15	<0.001
FeM-UF	0.88	0.99	-47.68	0.05
DeM-UF	-13.81	0.44	-70	<0.01
DeM-FeM	-14.69	0.37	-22.32	0.60

692 Tukey HSD *post–hoc* pairwise comparison among treatments for guppies in Experiment 2. UM:
693 untreated males, UF: untreated females, DeM: males under demasculinisation, and FeM: males
694 under feminisation. A negative difference indicates that the second group in a treatment pair had
695 a higher parasite load than the first treatment.

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698 **Table 4:** Guppy survival by treatment post-infection with *Gyrodactylus turnbulli.*

Coefficient	Estimate	SEM	Z-value	P (>|z|)
Untreated females	-2	0.47	-4.3	<0.001
Feminisation males	-1.16	0.37	-3.1	0.002
Demasculinisation males	-1.48	0.4	-3.67	<0.001

699 Cox proportional hazards results for survival until Day 13 after infection, "day of mortality" as a
700 response variable, and "treatment" as explanatory variable. Values are for individuals of a given
701 treatment compared to the untreated males.

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