1 **A neglected fish stressor: mechanical disturbance during transportation impacts susceptibility to**

2 **disease in a globally important ornamental fish**

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4 Running page head: Fish transport influences disease susceptibility

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10 **ABSTRACT:** The transport of fish in aquaculture and the ornamental trade exposes fish to multiple

11 stressors that can cause mass mortalities and economic loss. Previous research on fish transport has

12 largely focused on chemical stress related to deterioration in water quality. Mechanical disturbance

13 during routine fish transport, however, is unpredictable and is a neglected potential stressor when

14 studying fish welfare. Stress induced immunosuppression, caused by mechanical disturbance can

15 increase the chances of contracting infections and significantly increase infection burden. Here, using

16 the model guppy-*Gyrodactylus turnbulli* host-parasite system and a new method of bagging fish

17 (Breathing Bags ™), which reduces mechanical disturbance during fish transport, we investigated

18 how parasite infections contracted after simulated transport impact infection trajectories on a

19 globally-important ornamental, freshwater species. Guppies exposed to mechanical transport

20 disturbance suffered significantly higher parasite burden compared to fish that did not experience

21 transport disturbance. Unfortunately, there was no significant reduction in parasite burden of fish

22 transported in the Breathing Bags ™ compared to standard polythene carrier bags. Thus, transport

23 induced mechanical disturbance, hitherto neglected as a stressor, can be detrimental to disease

24 resistance and highlights the need for specific management procedures to reduce the impact of

25 infectious diseases following routine fish transport.

26

27 **KEY WORDS**: Transport stress . mechanical disturbance . disease susceptibility . ornamental fish .

28 guppy . *Gyrodactylus turnbulli*

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30 ## 1. INTRODUCTION

31   For the animal industry, transportation can lead to maladaptive traits, including reduced

32 feeding, altered immune response and mortality (Cattle: Stockman et al. 2013, Swine: Zou et al. 2017,

33 Poultry: Matur et al. 2016, Fish: Momoda et al. 2007, Castro et al. 2016). Although the impact of

34 transport stress is a general animal welfare issue, priority of research has been placed on terrestrial

35 livestock (Schwartzkopf-Genswein et al. 2012) over aquatic species. Furthermore, current research

36 on transport stress in fish focusses on food fish and neglects the ornamental trade (Ashley 2007,

37 Stevens et al. 2017) despite fish being the most abundant pet in western households (American Pet

38 Products Association 2012). Indeed, with over 4500 freshwater fish and 1450 marine fish species

39 traded globally as pets, the ornamental trade is a lucrative business valued at U.S. $800 million to

40 $30 billion annually (Stevens et al. 2017) and this demand for ornamentals is increasing with

41 expansion of the global pet trade (Saxby et al. 2010). Increased fish transport is an inevitable

42 consequence of rising demands for exotic species and emphasis on meeting these demands includes

43 minimising transport costs which may lead to fish being transported in sub-optimal conditions.

44   Stressors experienced by fish during transport can lead to immunosuppression, with the

45 proposed mechanism linked to the release of catecholamines and glucocorticoid hormones as a

46 stress response (Barton 2002, Ackerman et al. 2006), which may increase disease susceptibility

47 (Caruso et al. 2002, Ramsay et al. 2009). There tends to be huge variability in immune responses to

48   stressors (see Tort 2011 for review), with chronic or acute stressors suppressing or enhancing

49   immunity (Dhabbar 2000). In Atlantic salmon, for example, chronic stress suppresses transcriptional

50   immune responses to pathogenic challenge, whereas acute stress enhanced it (Webster et al. 2018).

51   Thus, with transport stressors that remain under the radar, we are still in the dark as to how

52   pathogens will be affected by the host's immune response. Further complications arise when

53   variations in susceptibility to disease are linked to both host and pathogen species making the

54   outcome of transportation on fish welfare uncertain. Chinook salmon (*Oncorhynchus tshawytscha*)

55   and ayu (*Plecoglossus altivelis*), for example, exposed to transport conditions showed increased

56   susceptibility to bacterial infections (Iguchi et al. 2003, Ackerman et al. 2006). Channel

57   catfish (*Ictalurus punctatus)*, that experienced low water crowding stress as part of simulated

58   transport conditions, only showed increased susceptibility when exposed to *Ichthyophthirius*

59   *multifiliis*, but not to inoculation with the channel catfish virus (Davis et al. 2002).

60   Typically, fingerlings, juveniles and small fish are transported in plastic bags, filled with 25-

61   30% water and 70-75% air or pure oxygen (Carneiro & Urbinati 2001, Conte 2004). Presence of air

62   pockets in polythene bags for fish increases the chances of mechanical stress due to water

63   movement. In mechanical terms, stress is defined as a force applied across a surface per unit area for

64   all orientations of that surface (Chen & Han 2007). In addition, accumulation of carbon dioxide from

65   respiring fish can lead to displaced available oxygen, especially if stocking densities are high (Conte

66   2004). Thus, traditional transport carriers can expose fish to multiple stressors, including capture,

67   handling, overcrowding, abrupt changes in temperature and physical trauma (Robertson et al. 1988,

68   Portz et al. 2006). A decline in water quality caused by the accumulation of ammonia (Ackerman et

69   al. 2006), fluctuations in dissolved oxygen and pH (Moran et al. 2008, Sampaio & Freire 2016) which

70   are known fish stressors, is another consequence of transportation (Patterson et al. 2003,). Micro-

71   porous transport bags (Breathing Bags ™, Kordon ®) unlike traditional polythene bags allow

exchange of respired carbon dioxide with atmospheric oxygen. Being porous means the bags can be

completely filled with water (without the need to add air or oxygen) and since water is

incompressible relative to air, this should provide natural cushioning against mechanical stress for

fish being transported (Thiagarajan et al. 2011). The impact of other stressors associated with fish

transport has been previously investigated (water quality: Ackerman et al. 2006; Dhanasiri et al.

2011, capture and handling: Caruso et al. 2002, Thompson et al. 2016, stocking densities: Ramsay et

al. 2009) whereas mechanical stress has thus far remained neglected.

The transport procedure for fish varies globally depending on local animal trading laws and

whether fish are transported locally or internationally. The latter routinely involves fish quarantine

procedures before transport and border inspections post-arrival (Portz et al. 2006). In addition, such

fish will experience extended transport disturbance including multiple handlings due to inspections.

Fish transportation procedures typically lack routine screening procedures for parasites and

therefore represent a wide-scale welfare issue (Ashley 2007, Stevens et al. 2017). Ornamentals

transported from the wild or local pet shops may be reservoirs of undiagnosed infections that

become more pernicious due to stress-imposed immunosuppression following transport (Bonga

1997). Due to mixing of species from different geographic regions, disease dynamics in wholesalers,

retailers and hobbyist aquaria may result in parasite host switching and increased virulence (Kelly et

al. 2009). Accidental or intentional introduction of exotic species into local fish populations can cause

transmission of highly virulent parasites to which native fish species may be especially susceptibile

(Smit et al. 2017).

Ornamental fish trade practices routinely involve the addition of antiparasitic chemicals into

water and removal of weak or diseased fish which reduces disease outbreaks and keep parasite

numbers to a minimum (Stevens et al. 2017). Diseases with distinctive symptoms, such as those

caused by *Ichthyophthirius multifiliius* or *Saprolegnia parasitica*, are relatively easy to detect through

96 visual inspection of fish, leading to either quarantine or euthanizing infected individuals to halt

97 spread of infections (FAO 2012, Stentiford et al. 2017). Such standard practice for fish farmers and

98 hobbyists does reduce maintenance cost and for legal reasons many countries only sell or display fish

99 that appear healthy (Washington & Ababouch 2011). However, many parasites at low levels of

100 infection do not affect fish phenotype, making them undetectable to non-specialists. Ectoparasites,

101 such as *Gyrodactylus* species, typically require thorough microscopic examination to determine

102 parasite burden (Maceda-Veiga & Cable 2018), which is not a routine procedure for fish at any point

103 in the aquaculture or ornamental trade. For gyrodactylosis, there is no 100% effective treatment and

104 parasites can remain at low frequencies in fish populations that are being transported and then in

105 favourable conditions they can increase exponentially until stock survival is severely affected (Cable

106 2011). Thus, even if species harbour low-level infections due to the presence of anti-parasitic

107 chemicals, stressful transport conditions can sufficiently weaken the immune system allowing large

108 infection sources to be established in healthy stocks.

109 Amongst the most popular tropical fish species is the guppy (*Poecilia reticulata*, see Maceda-

110 Veiga 2016), which has been transported worldwide as an ornamental and biological control agent,

111 with 41 recorded introductions outside its native habitat (Magurran 2005). The most common

112 parasites of wild and ornamental guppies are viviparous monogenean *Gyrodactylus* spp. known for

113 their 'Russian doll' reproduction and direct transmission (Cable 2011). This makes them capable of

114 rapidly colonising a fish population, affecting their behaviour, including courtship, feeding and

115 shoaling (Kennedy et al. 1987, Kolluru et al. 2009, Hockley et al. 2014) and survival (Cable & van

116 Oosterhout 2007, Yamin et al. 2017).

117 Here we investigated the impact of simulated transportation on fish infection dynamics.

118 Specifically, we assessed how mechanical disturbance associated with traditional polythene carrier

119 material impacts susceptibility to disease in fish exposed to parasites after simulated transport. In

120 addition, we tested the efficacy of Breathing Bags ™ in helping alleviate mechanical disturbance-

121 induced elevated disease susceptibility and mortality.

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123 ## 2. MATERIALS AND METHODS

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125 ### 2.1. Host and parasite species maintenance

126 Male guppies (standard length: 12.1-17.4 mm) bred from a stock originating in the Lower

127 Aripo River in Trinidad, were initially housed at Exeter University before being transferred to Cardiff

128 University in October 2014. Guppies were kept in 70 L breeding tanks, containing artificial plants and

129 refugia. They were maintained under a 12 h light: 12 h dark photoperiod (lights on 07:00-19:00) at

130 $24 \pm 1°C$ and fed daily on dry food flake (Aquarium ®) and every alternate day on live freshly hatched

131 *Artemia* nauplii. Experimental infections utilized the Gt3 strain of *Gyrodactylus turnbulli*, isolated

132 from a Nottingham aquarium shop in October 1997 and subsequently maintained at Cardiff

133 University since 1999 on inbred guppies prior to this study. All work was approved by the Cardiff

134 University Animal Ethics Committee and conducted under UK Home Office licence PPL 303424.

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136 ### 2.2. Experimental design

137 To test the impact of traditional polythene bags versus Breathing Bags ™ on fish susceptibility

138 to disease, guppies (20 per experimental treatment) were experimentally infected after experiencing

139 simulated transport. All guppies were netted carefully from breeding tanks to minimise handling

140 stress and transferred to separate tanks for a 24 h holding period. Fish were not fed for the holding

141 period to ensure a post-absorptive stage and to minimise build-up of nitrogenous waste, as per

142 standard aquacultural practice (Berka 1986). To simulate transport stress, fish were randomly

143 allocated into either 48 x 21 cm polythene bag treatments (provided by Aquatic World, Cardiff) or

144 36 x 19 cm Breathing Bags™ treatments. The polythene bags were filled with one-third dechlorinated

145 water to two-thirds air which is the most common method of transporting small fish in aquaculture

146 (Conte, 2004). Air was not added to the Breathing Bags ™ as per supplier instructions to reduce

147 mechanical disturbance due to sloshing (Thiagarajan et al. 2011). Fish stocking density was 4 fish/l

148 for both bag treatments, which falls within approved guidelines for tropical freshwater species

149 stocking densities (OATA 2008) and each bag contained water volumes of up to 1.5l. To prevent

150 handling fish with nets, they were placed into bags while fully submerged. Bagged fish were then

151 contained in an insulated sealed thermal box (dimensions: 30 x 24 x 19 cm, 24±1°C) and placed onto

152 an orbital shaker (Stuart®) for 24 h at 50 rpm to simulate transport motion. The rotator allowed for

153 orbital movement on a horizontal platform, similar to any flat surface fish would be placed on in a

154 transport vehicle or aircraft (Portz et al. 2006). Control fish (n=20) were kept in bags without turning

155 on the orbital rotator, adjacent to an operating rotator to ensure fish were exposed to the same noise

156 levels.

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158   **2.3.   Experimental infections**

159   To perform controlled infections, guppies were lightly anaesthetised with 0.02% MS222 and

160 each fish was infected with two gyrodactylid worms. Parasite transfer was conducted using a

161 dissection microscope with fibre optic illumination following standard methods of King and Cable

162 (2007). Briefly, two worms from heavily infected donor fish were transferred to the caudal fin of

163 recipient hosts by placing the anaesthetised donor fish in close proximity to an anaesthetised naïve

164 host with the transfer monitored continuously using the dissecting microscope. Parasite infections

165 were then monitored every 48 h by anesthetising fish and the total number of gyrodactylids counted

166 over the first 17 days of infection. At Day 17, all fish that survived were treated with Levamisole

167 (Norbrook ®, UK) according to Schelkle et al. (2009) and their post-treatment recovery and any

168 further mortalities monitored for 3 weeks.

169

2.4. Water quality

171 As water quality can impact disease susceptibility (Ackerman et al. 2006), we measured water

172 ammonia (freshwater master test kit, API ®), pH (battery powered checker HANNA ®) and oxygen

173 saturation (dissolved oxygen meter, Lutron Electric Enterprise CO., LTD.) to ensure this did not vary

174 between treatment and control groups (n=5 bags per experiment). All water quality levels within the

175 polythene bags and Breathing Bags ™ post-transport were within normal ranges (ammonia levels

176 undetectable for both bag treatments), pH (pH 7.1-7.8) and oxygen saturation (20.4-21.4 %) and

177 consistent between treatments (Fisher's Exact test: oxygen, p= 0.958, pH, p=0.909).

178

2.5. Statistical analysis

180 All statistical analyses were conducted using RStudio v2.1 (R Development Core Team, 2015).

181 *G. turnbulli* mean intensity for all experiments, was defined as the average number of worms on

182 infected hosts (Bush et al. 1997). A generalized linear mixed model (GLMM) with a negative binomial

183 error family in the MASS R package was used to analyse the relationship between transport

184 treatments (polythene bags and Breathing Bags ™) and mean parasite intensity. Host standard

185 length, bag type (polythene bags and Breathing Bags ™) and treatment (transport and no-transport)

186 were treated as fixed factors. As parasite intensity was recorded for each individual fish at different

187 days, 'Fish ID' and 'days since initial infection' was included as a random effect in the GLMM to avoid

188 pseudoreplication by incorporating repeated-measures. Fish length was included in the initial model

189 but was removed because the size range did not explain significant variation (Thomas et al. 2013).

190 Area under the curve (AUC) is a statistical parameter that provides a measure for analyzing infection

191   trajectories over time using the trapezoid rule (White 2011). Area under the curve was analysed using

192   a second GLMM with a negative binomial error family. Finally, we used a Generalised Linear Model

193   (GLM) to analyse how peak parasite day and maximum parasite count varied with treatment. For

194   analysing maximum parasite count we used a negative binomial error family with a log link function

195   and a gaussian error family with an identity link function for peak parasite day. All error families were

196   determined based on the lowest Akaike Information Criterion (AIC) value. A logistic regression was

197   used to analyse motality between transported and control fish and between bag types.

3. RESULTS

199   Parasite dynamics were influenced by fish simulated transport, with guppies being

200   transported suffering significantly higher mean parasite intensity than untransported control fish

201   (GLMM: Z= 2.51, SE=0.16, p=0.009; Fig. 1). The carrier type used to simulate transport (polythene

202   bags or Breathing Bags ^{TM}), however, did not affect the mean intensity between transported and

203   untransported fish (GLMM: Z= 2.51, SE= 0.15, p=0.19). Total infection trajectory over 17-days, as

204   measured through Area Under Curve (AUC) was significantly greater in fish that experienced

205   simulated transport versus controls (GLMM: Z=2.42, SE=0.17, p=0.01). Similarly, peak parasite day

206   (t=2.24, SE=0.25, p=0.02) and the associated maximum parasite count (Z=6.73, SE=0.06, p<0.001)

207   were significantly different between transported and control fish: with maximum parasite count on

208   peak days having approximately 51% greater parasite load compared to controls in both carrier types

209   (Breathing bags^{TM}= 50.9% greater, polythene bags= 51.2% greater). There was no significant

210   difference in mortality between simulated transport and control fish within the same bags or

211   between Breathing Bags^{TM} and polythene bags (between bags, GLM: Z =0.18 ,SE=0.35, p= 0.85; within

212   same bags, GLM: Z = 0.89, SE= 0.36, p= 0.371).

213

4. DISCUSSION

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215   Simulated transport significantly affected guppy susceptibility to infections with *Gyrodactylus*

216 *turnbulli* showing for the first time the impact of mechanical disturbance on disease dynamics.

217 Unfortunately, increased parasite burdens were not ameliorated following use of specialized

218 Breathing Bags ™ even though these bags did reduce the level of water sloshing during mechanical

219 disturbance; although it should be noted that there could be additional benefits of these bags, not

220 tested here, for example in terms of maintaining higher water quality. Mechanical stress is a broad

221 term that encompasses aspects of physical forces such as pressure and impulse (Thiagarajan et al.

222 2011) and reduced slosh does not rule out the possibility of such forces acting on fish within

223 Breathing Bags™ during the transport simulation. Thus, fish transported in Breathing Bags™ may

224 indeed have experienced a form of mechanical stress despite reduced water sloshing leading to

225 increased susceptibility to disease.

226   The link between stressors and susceptibility to disease in fish is influenced by the production

227 of cortisol, which is an immunosuppressant (Tort et al. 2003, Tort 2011). While the relationship

228 between a stress event and immunosuppression is far from clear (reviewed by Tort 2011), the

229 transport process for fish is associated with multiple stressors including handling and netting, with

230 water quality deterioration considered the major stressor linked to high stocking density (see Braun

231 & Nuñer 2014), which has been implicated in elevated cortisol levels, increased disease susceptibility

232 and significant mortality levels (Caruso et al. 2002, Iguchi et al. 2003, Cho et al. 2009, Robertson et

233 al. 2017). However, for the current study fluctuating water quality, temperature, lighting, noise,

234 netting and stocking densities were controlled, leaving mechanical disturbance as the major stressor.

235 While we are unaware of how long a stress response would last in guppies post-transport, as there

236 is likely a species level difference in cortisol production (Honryo et al. 2018), mechanical disturbance

237 in our transported guppies could have caused elevated cortisol production during a stress response

238   leading to immunosuppression. Surprisingly, guppies exposed to gyrodactylid infection immediately

239   prior to experiencing mechanical transport disturbance did not show a significant effect of

240   transportation on total infection infection trajectories or mean parasite intensity compared to

241   untransported fish (Appendix), which indicates immune status at the time of initial infection is the

242   most important factor determining disease outcome.

243   Undiagnosed infections on imported fish are a major biosecurity risk in the ornamental trade

244   (Maceda-Veiga & Cable 2018), particularly as they may introduce novel parasite species to which

245   local hosts have no immunity (Paterson et al. 2012). The current study emphasises the need for

246   stricter screening procedures after transport, as diseases such as gyrodactylosis are difficult to

247   diagnose without thorough microscopic screening and can cause an explosion in parasite burden due

248   to transport stress. Application of anesthetic agents, like clove oil and MS-222, into water prior to

249   transport has shown limited efficacy in reducing stress and mortality in transported fish (Rubec et al.

250   2000) and actually is associated with the risk of respiratory failure (Wagner et al. 2003, Pramod et al.

251   2010). In contrast, addition of compounds, such as salt, prior to fish transportation, can reduce

252   transport-related mortality (Oyoo-Okoth et al. 2011); however, they have variable efficacy on

253   diseases such as gyrodactylosis, as treatment is often time, concentration and species dependant

254   (Schelkle et al. 2011). Studies of parasite diversity in the ornamental trade (pet shops, retailers and

255   home aquaria) highlight *Gyrodactylus* spp. as one of the most common group of parasites detected

256   during screening procedures (Trujillo-González et al. 2018, Maceda-Veiga & Cable 2018). Thus, the

257   impact of this monogenean infection remains a serious welfare issue for global ornamental trade.

258   For the first time our investigation highlights that even when water quality, stocking density and

259   temperature are stable, mechanical disturbance during transport, hitherto neglected as a potential

260   stressor, significantly impacts susceptibility to infections in fish. With disease remaining the major

261   factor limiting the expansion of global fish trade (FAO 2016), investigating stressors that have

262 remained under the radar thus far may prove crucial in a growing trend emphasizing the need for

263 improved fish welfare.

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548 **Fig. 1. Mechanical disturbance significantly impacted susceptibility to disease in *Poecilia reticulata***
549 ***exposed to *Gyrodactylus turnbulli* infections after experiencing 24h simulated transport in both***
550 **types of transport bags (Breathing Bags^{TM} and polythene bags)**. Standard Error bars slightly
551 transposed to one side to prevent overlap.
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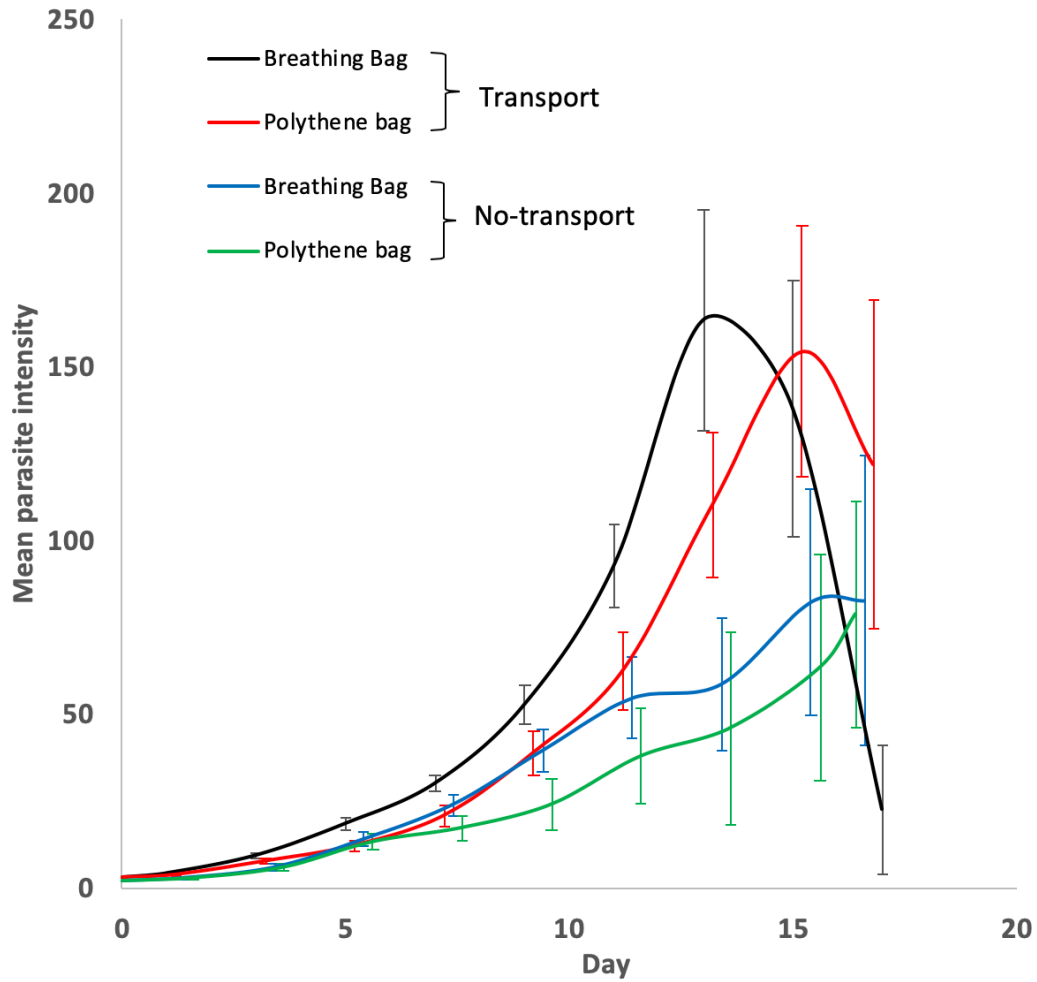
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567   **Appendix: Transport induced mechanical stress impact on infection trajectories of**

568   **guppies with pre-existing infections**

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570   Here, we investigated how mechanical disturbance during simulated transport impacted pre-existing

571   high burden infections in guppies (*Poecilia reticulata*).

572   **Materials and Methods**

573   Each guppy (n=20 per treatment) was experimentally infected with 30 *G. turnbulli* worms, packaged

574   in standard polythene bags and then exposed to simulated transport (as described in the Main Text)

575   or left as untransported controls. After 24h of simulated transport, all fish (transport and control)

576   were individually isolated in 1l pots and screened every 48h over 17 days to monitor their infection

577   trajectories and the data was analysed as described in the Main Text.

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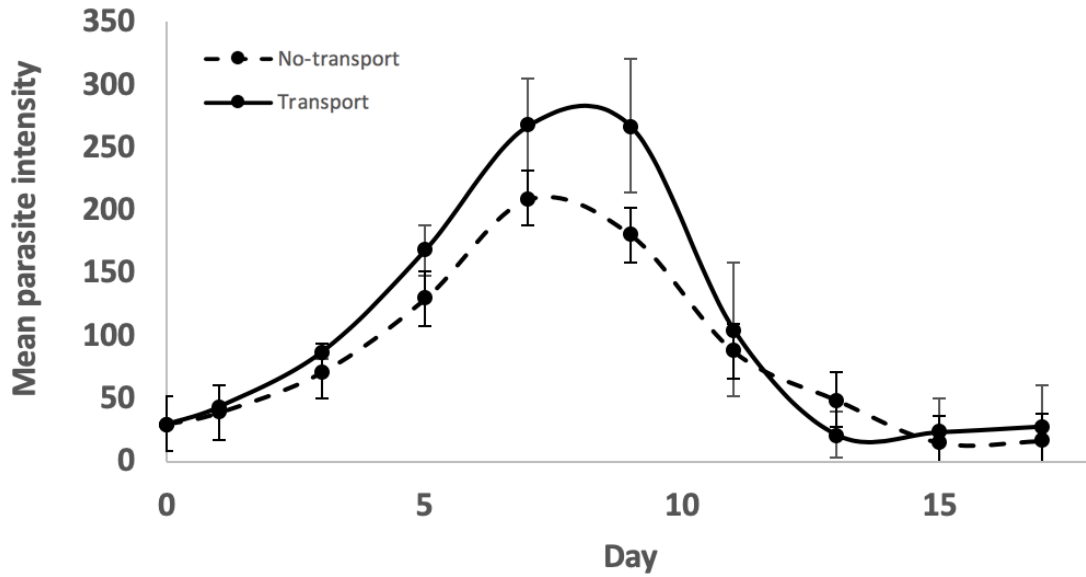
579   **Results**

580   Mean parasite intensity and total infection trajectories over 17 days as measured by AUC were not

581   significantly different between transported guppies and controls (mean parasite intensity: GLMM:

582   Z=1.64, SE= 0.1, p= 0.1; AUC: GLM: Z= 0.6, SE= 0.38, p=0.54, A.1.). Peak parasite burden, however,

583   was significantly higher in guppies that experienced simulated transport (GLM: Z=2.72, SE= 0.05,

584   p=0.006) and timing of peak parasite burden was also earlier in these guppies compared to controls

585   (GLM: t=-3.83, SE= 0.03, p=0.0001).

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589   **A.1. Infection trajectory with standard error bars showing *Poecilia reticulata* exposed to a starting**
590   **point *Gyrodactylus turnbulli* infection of 30 worms prior to transport did not suffer significantly**
591   **elevated mean parasite intensity compared to controls.**
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