A neglected fish stressor: mechanical disturbance during transportation impacts susceptibility to disease in a globally important ornamental fish

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ABSTRACT: The transport of fish in aquaculture and the ornamental trade exposes fish to multiple stressors that can cause mass mortalities and economic loss. Previous research on fish transport has largely focused on chemical stress related to deterioration in water quality. Mechanical disturbance during routine fish transport, however, is unpredictable and is a neglected potential stressor when studying fish welfare. Stress induced immunosuppression, caused by mechanical disturbance can increase the chances of contracting infections and significantly increase infection burden. Here, using the model guppy-*Gyrodactylus turnbulli* host-parasite system and a new method of bagging fish (Breathing Bags ™), which reduces mechanical disturbance during fish transport, we investigated how parasite infections contracted after simulated transport impact infection trajectories on a globally-important ornamental, freshwater species. Guppies exposed to mechanical transport disturbance suffered significantly higher parasite burden compared to fish that did not experience transport disturbance. Unfortunately, there was no significant reduction in parasite burden of fish transported in the Breathing Bags ™ compared to standard polythene carrier bags. Thus, transport induced mechanical disturbance, hitherto neglected as a stressor, can be detrimental to disease resistance and highlights the need for specific management procedures to reduce the impact of infectious diseases following routine fish transport.

KEY WORDS: Transport stress . mechanical disturbance . disease susceptibility . ornamental fish . guppy . *Gyrodactylus turnbulli*

1. INTRODUCTION

For the animal industry, transportation can lead to maladaptive traits, including reduced feeding, altered immune response and mortality (Cattle: Stockman et al*.* 2013, Swine: Zou et al. 2017, Poultry: Matur et al*.* 2016, Fish: Momoda et al*.* 2007, Castro et al. 2016). Although the impact of transport stress is a general animal welfare issue, priority of research has been placed on terrestrial livestock (Schwartzkopf-Genswein et al. 2012) over aquatic species. Furthermore, current research on transport stress in fish focusses on food fish and neglects the ornamental trade (Ashley 2007, Stevens et al. 2017) despite fish being the most abundant pet in western households (American Pet Products Association 2012). Indeed, with over 4500 freshwater fish and 1450 marine fish species traded globally as pets, the ornamental trade is a lucrative business valued at U.S. $800 million to $30 billion annually (Stevens et al. 2017) and this demand for ornamentals is increasing with expansion of the global pet trade (Saxby et al. 2010). Increased fish transport is an inevitable consequence of rising demands for exotic species and emphasis on meeting these demands includes minimising transport costs which may lead to fish being transported in sub-optimal conditions.

Stressors experienced by fish during transport can lead to immunosuppression, with the proposed mechanism linked to the release of catecholamines and glucocorticoid hormones as a stress response (Barton 2002, Ackerman et al. 2006), which may increase disease susceptibility (Caruso et al. 2002, Ramsay et al. 2009). There tends to be huge variability in immune responses to stressors (see Tort 2011 for review), with chronic or acute stressors suppressing or enhancing immunity (Dhabbar 2000). In Atlantic salmon, for example, chronic stress suppresses transcriptional immune responses to pathogenic challenge, whereas acute stress enhanced it (Webster et al. 2018). Thus, with transport stressors that remain under the radar, we are still in the dark as to how pathogens will be affected by the host's immune response. Further complications arise when variations in susceptibility to disease are linked to both host and pathogen species making the outcome of transportation on fish welfare uncertain. Chinook salmon (*Oncorhynchus tshawytscha*) and ayu (*Plecoglossus altivelis*), for example, exposed to transport conditions showed increased susceptibility to bacterial infections (Iguchi et al. 2003, Ackerman et al. 2006). Channel catfish (*Ictalurus punctatus)*, that experienced low water crowding stress as part of simulated transport conditions, only showed increased susceptibility when exposed to *Ichthyophthirius multifiliis*, but not to inoculation with the channel catfish virus (Davis et al. 2002).

Typically, fingerlings, juveniles and small fish are transported in plastic bags, filled with 25-30% water and 70-75% air or pure oxygen (Carneiro & Urbinati 2001, Conte 2004). Presence of air pockets in polythene bags for fish increases the chances of mechanical stress due to water movement. In mechanical terms, stress is defined as a force applied across a surface per unit area for all orientations of that surface (Chen & Han 2007). In addition, accumulation of carbon dioxide from respiring fish can lead to displaced available oxygen, especially if stocking densities are high (Conte 2004). Thus, traditional transport carriers can expose fish to multiple stressors, including capture, handling, overcrowding, abrupt changes in temperature and physical trauma (Robertson et al. 1988, Portz et al. 2006). A decline in water quality caused by the accumulation of ammonia (Ackerman et al. 2006), fluctuations in dissolved oxygen and pH (Moran et al. 2008, Sampaio & Freire 2016) which are known fish stressors, is another consequence of transportation (Patterson et al. 2003,). Micro-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 visual inspection of fish, leading to either quarantine or euthanizing infected individuals to halt spread of infections (FAO 2012, Stentiford et al. 2017). Such standard practice for fish farmers and hobbyists does reduce maintenance cost and for legal reasons many countries only sell or display fish that appear healthy (Washington & Ababouch 2011). However, many parasites at low levels of infection do not affect fish phenotype, making them undetectable to non-specialists. Ectoparasites, such as *Gyrodactylus* species, typically require thorough microscopic examination to determine parasite burden (Maceda-Veiga & Cable 2018), which is not a routine procedure for fish at any point in the aquaculture or ornamental trade. For gyrodactylosis, there is no 100% effective treatment and parasites can remain at low frequencies in fish populations that are being transported and then in favourable conditions they can increase exponentially until stock survival is severely affected (Cable 2011). Thus, even if species harbour low-level infections due to the presence of anti-parasitic chemicals, stressful transport conditions can sufficiently weaken the immune system allowing large infection sources to be established in healthy stocks.

Amongst the most popular tropical fish species is the guppy (*Poecilia reticulata*, see Maceda-Veiga 2016), which has been transported worldwide as an ornamental and biological control agent, with 41 recorded introductions outside its native habitat (Magurran 2005). The most common parasites of wild and ornamental guppies are viviparous monogenean *Gyrodactylus* spp. known for their 'Russian doll' reproduction and direct transmission (Cable 2011). This makes them capable of rapidly colonising a fish population, affecting their behaviour, including courtship, feeding and shoaling (Kennedy et al. 1987, Kolluru et al. 2009, Hockley et al. 2014) and survival (Cable & van Oosterhout 2007, Yamin et al. 2017).

Here we investigated the impact of simulated transportation on fish infection dynamics. Specifically, we assessed how mechanical disturbance associated with traditional polythene carrier material impacts susceptibility to disease in fish exposed to parasites after simulated transport. In addition, we tested the efficacy of Breathing Bags ™ in helping alleviate mechanical disturbance-induced elevated disease susceptibility and mortality.

2. MATERIALS AND METHODS

2.1. Host and parasite species maintenance

Male guppies (standard length: 12.1-17.4 mm) bred from a stock originating in the Lower Aripo River in Trinidad, were initially housed at Exeter University before being transferred to Cardiff University in October 2014. Guppies were kept in 70 L breeding tanks, containing artificial plants and refugia. They were maintained under a 12 h light: 12 h dark photoperiod (lights on 07:00-19:00) at 24 ± 1°C and fed daily on dry food flake (Aquarium ®) and every alternate day on live freshly hatched *Artemia* nauplii. Experimental infections utilized the Gt3 strain of *Gyrodactylus turnbulli*, isolated from a Nottingham aquarium shop in October 1997 and subsequently maintained at Cardiff University since 1999 on inbred guppies prior to this study. All work was approved by the Cardiff University Animal Ethics Committee and conducted under UK Home Office licence PPL 303424.

2.2. Experimental design

To test the impact of traditional polythene bags versus Breathing Bags ™ on fish susceptibility to disease, guppies (20 per experimental treatment) were experimentally infected after experiencing simulated transport. All guppies were netted carefully from breeding tanks to minimise handling stress and transferred to separate tanks for a 24 h holding period. Fish were not fed for the holding period to ensure a post-absorptive stage and to minimise build-up of nitrogenous waste, as per standard aquacultural practice (Berka 1986). To simulate transport stress, fish were randomly allocated into either 48 x 21 cm polythene bag treatments (provided by Aquatic World, Cardiff) or

36 x 19 cm Breathing Bags™ treatments. The polythene bags were filled with one-third dechlorinated water to two-thirds air which is the most common method of transporting small fish in aquaculture (Conte, 2004). Air was not added to the Breathing Bags ™ as per supplier instructions to reduce mechanical disturbance due to sloshing (Thiagarajan et al. 2011). Fish stocking density was 4 fish/l for both bag treatments, which falls within approved guidelines for tropical freshwater species stocking densities (OATA 2008) and each bag contained water volumes of up to 1.5l. To prevent handling fish with nets, they were placed into bags while fully submerged. Bagged fish were then contained in an insulated sealed thermal box (dimensions: 30 x 24 x 19 cm, 24±1°C) and placed onto an orbital shaker (Stuart®) for 24 h at 50 rpm to simulate transport motion. The rotator allowed for orbital movement on a horizontal platform, similar to any flat surface fish would be placed on in a transport vehicle or aircraft (Portz et al. 2006). Control fish (n=20) were kept in bags without turning on the orbital rotator, adjacent to an operating rotator to ensure fish were exposed to the same noise levels.

2.3. Experimental infections

To perform controlled infections, guppies were lightly anaesthetised with 0.02% MS222 and each fish was infected with two gyrodactylid worms. Parasite transfer was conducted using a dissection microscope with fibre optic illumination following standard methods of King and Cable (2007). Briefly, two worms from heavily infected donor fish were transferred to the caudal fin of recipient hosts by placing the anaesthetised donor fish in close proximity to an anaesthetised naïve host with the transfer monitored continuously using the dissecting microscope. Parasite infections were then monitored every 48 h by anesthetising fish and the total number of gyrodactylids counted over the first 17 days of infection. At Day 17, all fish that survived were treated with Levamisole

(Norbrook ®, UK) according to Schelkle et al. (2009) and their post-treatment recovery and any further mortalities monitored for 3 weeks.

2.4. Water quality

As water quality can impact disease susceptibility (Ackerman et al. 2006), we measured water ammonia (freshwater master test kit, API ®), pH (battery powered checker HANNA ®) and oxygen saturation (dissolved oxygen meter, Lutron Electric Enterprise CO., LTD.) to ensure this did not vary between treatment and control groups (n=5 bags per experiment). All water quality levels within the polythene bags and Breathing Bags ™ post-transport were within normal ranges (ammonia levels undetectable for both bag treatments), pH (pH 7.1-7.8) and oxygen saturation (20.4-21.4 %) and consistent between treatments (Fisher's Exact test: oxygen, p= 0.958, pH, p=0.909).

2.5. Statistical analysis

All statistical analyses were conducted using RStudio v2.1 (R Development Core Team, 2015). *G. turnbulli* mean intensity for all experiments, was defined as the average number of worms on infected hosts (Bush et al. 1997). A generalized linear mixed model (GLMM) with a negative binomial error family in the MASS R package was used to analyse the relationship between transport treatments (polythene bags and Breathing Bags ™) and mean parasite intensity. Host standard length, bag type (polythene bags and Breathing Bags ™) and treatment (transport and no-transport) were treated as fixed factors. As parasite intensity was recorded for each individual fish at different days, 'Fish ID' and 'days since initial infection' was included as a random effect in the GLMM to avoid pseudoreplication by incorporating repeated-measures. Fish length was included in the initial model but was removed because the size range did not explain significant variation (Thomas et al. 2013). Area under the curve (AUC) is a statistical parameter that provides a measure for analyzing infection trajectories over time using the trapezoid rule (White 2011). Area under the curve was analysed using a second GLMM with a negative binomial error family. Finally, we used a Generalised Linear Model (GLM) to analyse how peak parasite day and maximum parasite count varied with treatment. For analysing maximum parasite count we used a negative binomial error family with a log link function and a gaussian error family with an identity link function for peak parasite day. All error families were determined based on the lowest Akaike Information Criterion (AIC) value. A logistic regression was used to analyse motality between transported and control fish and between bag types.

3. RESULTS

Parasite dynamics were influenced by fish simulated transport, with guppies being transported suffering significantly higher mean parasite intensity than untransported control fish (GLMM: Z= 2.51, SE=0.16, p=0.009; Fig. 1). The carrier type used to simulate transport (polythene bags or Breathing Bags ™), however, did not affect the mean intensity between transported and untransported fish (GLMM: Z= 2.51, SE= 0.15, p=0.19). Total infection trajectory over 17-days, as measured through Area Under Curve (AUC) was significantly greater in fish that experienced simulated transport versus controls (GLMM: Z=2.42, SE=0.17, p=0.01). Similarly, peak parasite day (t=2.24, SE=0.25, p=0.02) and the associated maximum parasite count (Z=6.73, SE=0.06, p<0.001) were significantly different between transported and control fish: with maximum parasite count on peak days having approximately 51% greater parasite load compared to controls in both carrier types (Breathing bags™= 50.9% greater, polythene bags= 51.2% greater). There was no significant difference in mortality between simulated transport and control fish within the same bags or between Breathing Bags™ and polythene bags (between bags, GLM: Z =0.18 ,SE=0.35, p= 0.85; within same bags, GLM: Z = 0.89, SE= 0.36, p= 0.371).

4. DISCUSSION

Simulated transport significantly affected guppy susceptibility to infections with *Gyrodactylus turnbulli* showing for the first time the impact of mechanical disturbance on disease dynamics. Unfortunately, increased parasite burdens were not ameliorated following use of specialized Breathing Bags ™ even though these bags did reduce the level of water sloshing during mechanical disturbance; although it should be noted that there could be additional benefits of these bags, not tested here, for example in terms of maintaining higher water quality. Mechanical stress is a broad term that encompasses aspects of physical forces such as pressure and impulse (Thiagarajan et al. 2011) and reduced slosh does not rule out the possibility of such forces acting on fish within Breathing Bags™ during the transport simulation. Thus, fish transported in Breathing Bags™ may indeed have experienced a form of mechanical stress despite reduced water sloshing leading to increased susceptibility to disease.

The link between stressors and susceptibility to disease in fish is influenced by the production of cortisol, which is an immunosuppressant (Tort et al. 2003, Tort 2011). While the relationship between a stress event and immunosuppression is far from clear (reviewed by Tort 2011), the transport process for fish is associated with multiple stressors including handling and netting, with water quality deterioration considered the major stressor linked to high stocking density (see Braun & Nuñer 2014), which has been implicated in elevated cortisol levels, increased disease susceptibility and significant mortality levels (Caruso et al. 2002, Iguchi et al. 2003, Cho et al. 2009, Robertson et al. 2017). However, for the current study fluctuating water quality, temperature, lighting, noise, netting and stocking densities were controlled, leaving mechanical disturbance as the major stressor. While we are unaware of how long a stress response would last in guppies post-transport, as there is likely a species level difference in cortisol production (Honryo et al. 2018), mechanical disturbance in our transported guppies could have caused elevated cortisol production during a stress response leading to immunosuppression. Surprisingly, guppies exposed to gyrodactylid infection immediately prior to experiencing mechanical transport disturbance did not show a significant effect of transportation on total infection infection trajectories or mean parasite intensity compared to untransported fish (Appendix), which indicates immune status at the time of initial infection is the most important factor determining disease outcome.

Undiagnosed infections on imported fish are a major biosecurity risk in the ornamental trade (Maceda-Veiga & Cable 2018), particularly as they may introduce novel parasite species to which local hosts have no immunity (Paterson et al. 2012). The current study emphasises the need for stricter screening procedures after transport, as diseases such as gyrodactylosis are difficult to diagnose without thorough microscopic screening and can cause an explosion in parasite burden due to transport stress. Application of anesthetic agents, like clove oil and MS-222, into water prior to transport has shown limited efficacy in reducing stress and mortality in transported fish (Rubec et al. 2000) and actually is associated with the risk of respiratory failure (Wagner et al. 2003, Pramod et al. 2010). In contrast, addition of compounds, such as salt, prior to fish transportation, can reduce transport-related mortality (Oyoo-Okoth et al. 2011); however, they have variable efficacy on diseases such as gyrodactylosis, as treatment is often time, concentration and species dependant (Schelkle et al. 2011). Studies of parasite diversity in the ornamental trade (pet shops, retailers and home aquaria) highlight *Gyrodactylus* spp. as one of the most common group of parasites detected during screening procedures (Trujillo-González et al. 2018, Maceda-Veiga & Cable 2018). Thus, the impact of this monogenean infection remains a serious welfare issue for global ornamental trade. For the first time our investigation highlights that even when water quality, stocking density and temperature are stable, mechanical disturbance during transport, hitherto neglected as a potential stressor, significantly impacts susceptibility to infections in fish. With disease remaining the major factor limiting the expansion of global fish trade (FAO 2016), investigating stressors that have remained under the radar thus far may prove crucial in a growing trend emphasizing the need for improved fish welfare.

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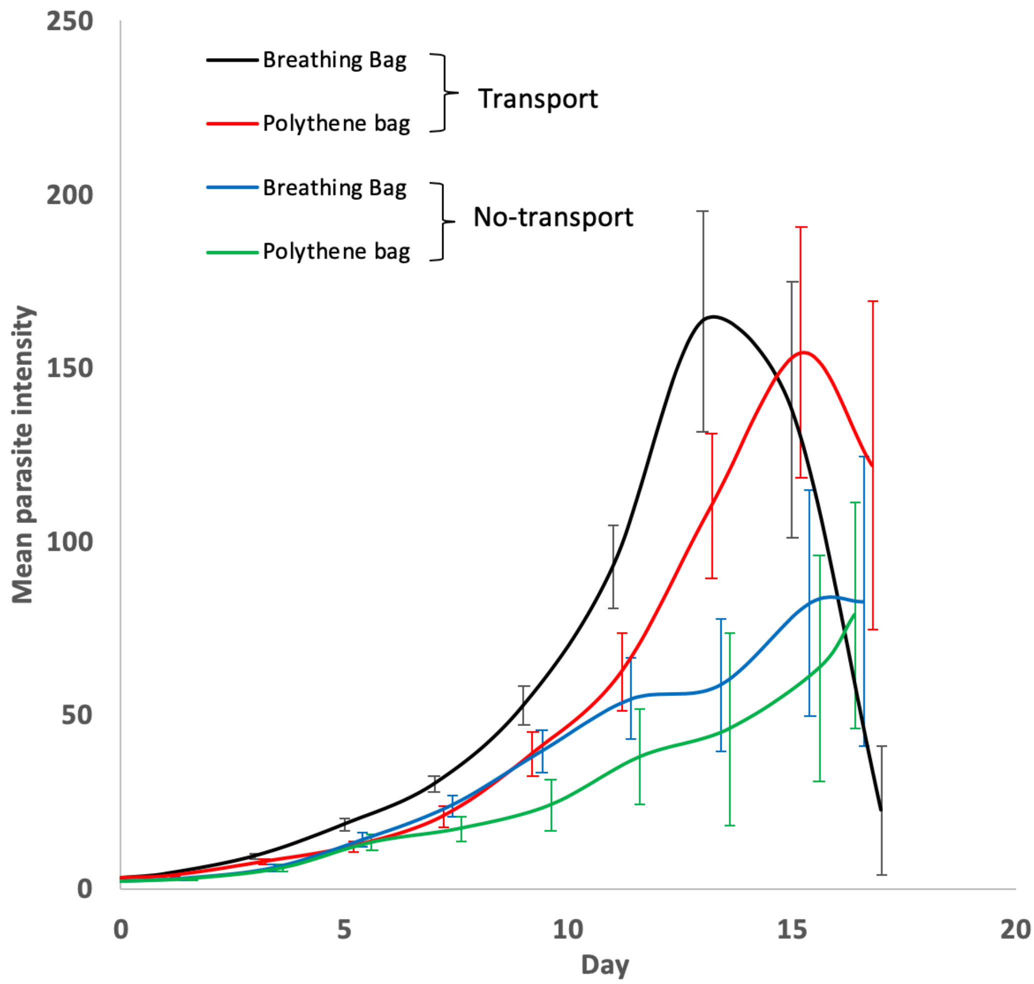
Fig. 1. Mechanical disturbance significantly impacted susceptibility to disease in *Poecilia reticulata* exposed to *Gyrodactylus turnbulli* infections after experiencing 24h simulated transport in both types of transport bags (Breathing Bags^{TM} and polythene bags). Standard Error bars slightly transposed to one side to prevent overlap.



Appendix: Transport induced mechanical stress impact on infection trajectories of guppies with pre-existing infections

Here, we investigated how mechanical disturbance during simulated transport impacted pre-existing high burden infections in guppies (*Poecilia reticulata*).

Materials and Methods

Each guppy (n=20 per treatment) was experimentally infected with 30 *G. turnbulli* worms, packaged in standard polythene bags and then exposed to simulated transport (as described in the Main Text) or left as untransported controls. After 24h of simulated transport, all fish (transport and control) were individually isolated in 1l pots and screened every 48h over 17 days to monitor their infection trajectories and the data was analysed as described in the Main Text.

Results

Mean parasite intensity and total infection trajectories over 17 days as measured by AUC were not significantly different between transported guppies and controls (mean parasite intensity: GLMM: $Z=1.64$, $SE= 0.1$, $p= 0.1$; AUC: GLM: $Z= 0.6$, $SE= 0.38$, $p=0.54$, A.1.). Peak parasite burden, however, was significantly higher in guppies that experienced simulated transport (GLM: $Z=2.72$, $SE= 0.05$, $p=0.006$) and timing of peak parasite burden was also earlier in these guppies compared to controls (GLM: $t=-3.83$, $SE= 0.03$, $p=0.0001$).

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