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1 Monitoring water quality changes and  
2 ornamental fish behaviour during  
3 commercial transport

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19 Live transport of fishes is recognised as a major source of stress leading to poor welfare and mortality  
20 within the ornamental fish industry. While previous studies have aimed to simulate the stressors  
21 experienced by fishes during transport in the laboratory, there is little documented evidence of the  
22 actual conditions experienced by fishes under commercial conditions. The aim of this study was to  
23 monitor water quality and fish health (physiology and behaviour) through a commercial supply chain  
24 for three popular freshwater ornamentals (neon tetra, oranda, variatus platy). Water samples were  
25 collected at nine stages of the supply chain beginning at arrival of the fishes at a UK wholesaler from  
26 Singapore through their recovery at the wholesaler, subsequent shipment to and recovery at retail  
27 stores. Water chemistry was recorded at various points in the transport chain and the water tested for  
28 common fish pathogens. Fish health parameters measured included mortality, injuries, waterborne  
29 cortisol and behavioural changes. Most water parameters were found to change significantly through  
30 the transport chain, including pH, carbonate hardness (KH), chloride (Cl<sup>-</sup>), nitrate (NO<sub>3</sub><sup>-</sup>), sodium  
31 (Na<sup>+</sup>), magnesium (Mg<sup>2+</sup>), potassium (K<sup>+</sup>) and calcium (Ca<sup>2+</sup>). Pathogens were detected in the water  
32 at each stage of transport, but no disease outbreaks were observed. Mortality was low and was not  
33 affected by transport stage. Neon tetras and orandas showed some behavioural changes during  
34 transport but the behaviour of variatus platys was more affected by transport stage. The findings of  
35 this study identify the changes in water quality experienced by fishes during commercial transport that  
36 are often over-looked in simulated studies and confirm the need for species-specific indicators of  
37 welfare during commercial transport.

38 Keywords: ornamental fishes, transport, welfare, behaviour, water chemistry, fish pathogens

39

40 Highlights

- 41 • First published study monitoring water quality and fish health (physiology and behaviour)  
42 through a commercial ornamental fish supply chain.
- 43 • Water quality was high overall but significant changes in water chemistry occurred between  
44 different stages of the supply chain.
- 45 • Potentially pathogenic microbes were detected in all samples but frequency of detection  
46 changed throughout the supply chain.
- 47 • Of the measured health indicators, behaviour was found to change the most between different  
48 stages of transport, and was also species-specific.

## 49 **1. Introduction**

50 It is estimated that over 2 billion live ornamental fishes are transported annually (Monticini,  
51 2010), with records of over 50 exporting countries and over 75 importing countries during 2012-2018  
52 (United Nations, 2019). Fish are either wild caught or farm-reared and undergo multiple transport  
53 journeys throughout the supply chain (Olivier, 2003; Cohen, Valenti and Calado, 2013). The duration  
54 of international transport of ornamentals is on average 24 h, but delays due to weather or customs  
55 procedures can cause an increase in transportation time (up to 72 h in some reported cases (Cole et al.,  
56 1999; Silva et al., 2015)). Within the UK, the majority of fish are imported from farms or wholesalers  
57 in developing countries in South-East Asia or South America (Leal et al., 2015). Transport can be a  
58 considerable source of stress in fish (Portz, Woodley and Cech, 2006; Sampaio and Freire, 2016),  
59 therefore legislation is in place to minimise the impact of live animal transport on welfare (e.g. Article  
60 3 of Council Regulation (EC) No. 1/2005, UK), with use of transport containers that are appropriate  
61 for the species and comply with transport regulations (e.g. International Air Transport Association  
62 Live Animals Regulations, Ch. II, 4.1, Defra, 2006).

63 Over the duration of the transport, the quality of the containment water can degrade, resulting in  
64 sub-optimal conditions for the fish and high mortality if chemical parameters are not controlled  
65 (Harmon, 2009; Lim et al., 2003). Studies simulating commercial transport have shown that the most  
66 important water parameters to manage are dissolved oxygen (DO), temperature, pH and ammonia  
67 (Bower and Turner, 1982; Harmon, 2009; Ramírez-duarte et al., 2013; Sampaio and Freire, 2016;  
68 Silva et al., 2015; Teo et al., 1989). In simulated transport studies, fish are contained in water  
69 originating from the same source before, during and after the transport and any fluctuation in water  
70 quality is caused by the fish in the transport bag. However, within commercial settings, ornamental  
71 fishes are exposed to water from many different sources from their time at either aquaculture farms or  
72 in the wild, to their final arrival in a home aquarium. The water quality at different stages of the  
73 transport chain is likely to change abruptly, not only due to excretion from the fish but also from  
74 different water sources used by the various stakeholders and any measures used to control water  
75 quality and health of the fish. Elements such as calcium ( $\text{Ca}^{2+}$ ), potassium ( $\text{K}^+$ ), magnesium ( $\text{Mg}^{2+}$ ),

76 and sodium (Na<sup>+</sup>) are important for osmoregulation (Baldisserotto et al., 2007; Evans, 2008) whereas  
77 aluminium (Al), copper (Cu) and lead (Pb) are toxic in excess to fishes (Shuhaimi-Othman et al.,  
78 2015; Spry and Wiener, 1991). Only a few studies have assessed changes in water quality and fish  
79 health for a complete transport chain (i.e. from the farm or time of capture to the retail store) and the  
80 range of water quality parameters measured in these studies was limited (Baldisserotto et al., 2014;  
81 Correia et al., 2011; Rodrigues et al., 2013).

82 Fish experiencing stress can become more susceptible to pathogenic microbes present in the water  
83 which can in turn result in a wide range of diseases (Crosby et al., 2005; Scholz, 1999; Yanong,  
84 2010). Common pathogens of ornamental fishes are *Mycobacterium* spp. (causative agents of acute  
85 and chronic mycobacteriosis and include *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. haemophilum*,  
86 *M. marinum*, *M. peregrinum*) (Gauthier and Rhodes, 2009; Prearo et al., 2004; Whipps et al., 2012),  
87 *Vibrio* spp. (causative agent of vibriosis, Thune, Stanley and Cooper, 1993; Roberts, Palmeiro and  
88 Weber, 2009), *Flavobacterium columnare* (causing columnaris disease, Decostere et al., 1999),  
89 *Pseudomonas* spp. (clinical signs of infection include ulcers, septicaemia, fin rot, red spot, Austin and  
90 Allen-Austin, 1985; Murphy and Lewbart, 1995), *Aeromonas* spp. (clinical signs include lesions,  
91 ulcers, tail or fin rot, haemorrhagic septicaemia, Citarasu et al., 2011; Roberts et al., 2009) and  
92 *Streptococcus* spp. (clinical signs include lethargy, poor appetite, erratic swimming, curved body  
93 shape, lesions, exophthalmia (also known as “pop-eye”), Hernández, Figueroa and Iregui, 2009; Amal  
94 and Zamri-Saad, 2011). Disease due to microbial pathogens is a widespread problem within the  
95 ornamental fish industry, and to our knowledge, no study has found the transport water of ornamental  
96 fishes to be pathogen-free. However, little research has been carried out assessing changes in the  
97 presence of pathogens at different stages of transport.

98 In addition to the lack of information on change in water quality during commercial transport,  
99 little is known about the cumulative effect of repeated transport and exposure to water of varying  
100 quality on the stress and welfare of ornamental fishes. Most studies assessing stress experienced in  
101 this context measure a range of stress indicators such as plasma cortisol, lactate and glucose levels  
102 (Ferreira et al., 2017; Inoue et al., 2005; Salbego et al., 2015) and the techniques involved are invasive

103 (Abreu et al., 2014; Azambuja et al., 2011; Zanuzzo et al., 2017). However, in small ornamentals,  
104 taking a blood sample in order to measure these indicators of stress often requires sacrificing the fish.  
105 Less invasive indicators that could potentially be used include levels of cortisol in the water (Ellis et  
106 al., 2004; Ruane and Komen, 2003), changes in behaviour such as increased aggression (Braithwaite  
107 and Ebbesson, 2014; Huntingford et al., 2006; Weber, 2011), increased ventilation rate as an indicator  
108 of increased metabolic rate (Brydges et al., 2009; Huntingford et al., 2006; Portz et al., 2006) and  
109 darkening of the fish skin and eye (Backström et al., 2015; Freitas et al., 2014; Huntingford et al.,  
110 2006). However, we could find no study that assessed transport stress in ornamental fishes using non-  
111 invasive sampling techniques on a commercial scale.

112 In order to refine commercial practices, there is a need to understand the way that water  
113 quality can change across the transport chain and how this may influence ornamental fish health and  
114 behaviour. The aim of this study was to monitor water quality, presence of waterborne pathogens and  
115 ornamental fish behaviour over the course of a commercial transport chain, from arrival at a UK  
116 aquatics wholesaler following air transport from Singapore, through to post-transport recovery at  
117 retail stores.

118

## 119 **2. Materials and Methods**

### 120 **2.1. Study Animals**

121 The commercial transport of three ornamental fish species was studied to assess changes in water  
122 chemistry, waterborne pathogens and fish physiology and behaviour. The three species investigated  
123 were neon tetra (*Paracheirodon innesi*), small oranda goldfish (*Carassius auratus*) and yellow strain  
124 variatus platy (*Xiphophorus variatus*). Ten bags of each species were randomly selected and sampled  
125 over a period of 10 weeks starting from the fishes' arrival at a UK wholesaler. The fishes were all  
126 reared in farms in Singapore and prior to transport to the UK food was withheld for 24 h prior before  
127 fish were packed in polyethylene bags (neon tetra: 20 x 16.5 x 70 cm; oranda and variatus platy: 35.5  
128 x 70 cm) containing water (neon tetra: 2.7 l; oranda: 1.4 l and variatus platy: 4 l) and 25 ml of liquid

129 zeolite with the air space at the top of the bag filled with pure oxygen. The stocking density was 148  
130 fish l<sup>-1</sup> for neon tetra, 72 fish l<sup>-1</sup> for oranda and 45 fish l<sup>-1</sup> for variatus platy. The bags were then air-  
131 tight sealed using a metal wire and placed into a polystyrene box insulated with newspapers along  
132 with a variable number of other bags, then into a cardboard box. The bags of fish were air-shipped to  
133 a UK airport where they went through customs and were then transported by road to an aquatics  
134 wholesaler. The total transport duration was approximately 32 h. On arrival at the wholesaler, water  
135 samples were taken from the bags for later analysis of water chemistry, waterborne pathogens and  
136 waterborne cortisol concentrations (see details below). Samples for water cortisol were frozen  
137 immediately on dry ice. Bags containing the remaining water and fish were then placed individually  
138 into aerated tanks (76 cm x 38 cm x 38 cm) containing 100 l of water at 24° C. At this point the  
139 antibiotic tetracycline was added to all the tanks (20 mg l<sup>-1</sup>) so that it was fully dissolved by the time  
140 the fish were added. The bags were floated in the tank for approximately 30 min to allow  
141 temperatures to equilibrate, and then the bags were opened and briefly submerged to introduce tank  
142 water into the bag. Approximately 30 min later the fish were released into the tank. During recovery  
143 at the wholesaler, fish were kept under a 10:14 light/dark regime with a light fitted with a UV filter.  
144 Fish were fed three times daily *ad libitum* with Vitalis tropical or goldfish flake (World Feeds Ltd,  
145 UK) by members of wholesaler staff, who at this point would also remove any dead fish. On day 2 of  
146 recovery at the wholesaler, methylene blue (0.1%) was added to all the tanks (1ml l<sup>-1</sup>). The fish then  
147 spent 7 days at the wholesaler, where water samples were taken on days 1, 2 and 5 for later analysis of  
148 water chemistry, prior to being transported to retail stores.

149 For transport to regional retail stores, the fish underwent the same packing process as for  
150 international transport, but no zeolite was added to the bag and food was not withheld prior to  
151 bagging. The loading density was 22 fish l<sup>-1</sup> for neon tetra, 7 fish l<sup>-1</sup> for oranda and 11 fish l<sup>-1</sup> for  
152 variatus platy. Water samples were taken from each bag before it was sealed for later analysis of  
153 water chemistry, waterborne pathogens and water cortisol concentrations. Ten bags of each species  
154 were transported by road to five different retail stores (each store received two bags per species over a  
155 period of 10 weeks), with a journey time of less than 6 h. On arrival at the retail store, water samples



156 were again taken from each bag for water chemistry and water cortisol. Each bag was then placed into  
157 a separate recovery tank, and the fishes were acclimated using the same process as used at the  
158 wholesaler. Fish were kept under a 12 h light/dark regime. They were fed twice daily *ad libitum* (neon  
159 tetra: Love Fish tropical fish flakes, oranda: Love Fish coldwater pellets by Pets at Home Group Plc,  
160 variatus platy: Love Fish temperate fish flakes) by retail store staff, who at this point also removed  
161 any dead fish. Water samples were taken on days 1, 2 and 5 of recovery at the stores for analysis of  
162 water chemistry and on day 5 only for analysis of waterborne pathogens. In addition to the collection  
163 of water samples, video recordings (30 min) were made using cameras (WiMiUS 4K 16MP Action)  
164 mounted on monopods and set at 720 P (progressive scan) and 120 fps (frames per second). This was  
165 carried out on a number of occasions: 1) upon arrival at the aquatics wholesaler, 2) on days 1, 2 and 5  
166 of recovery at the wholesaler, 3) before packing for transport to the retail stores, 4) on arrival at the  
167 retail stores and 5) on days 1, 2 and 5 of recovery at the stores.

## 168 2.2. Water Chemistry

169 The water pH, dissolved oxygen (DO) and temperature ( $^{\circ}$  C) were measured using a Hach  
170 HQ30d meter fitted with a Hach IntelliCAL<sup>TM</sup> pH probe, and a Hach LDO101 DO probe. Carbonate  
171 hardness (KH) was measured using a KH (d<sup>o</sup> KH) test solution kit (API<sup>®</sup> Mars Fishcare, Solution ID  
172 #3339). Water samples (50 ml) for chemistry were collected in a homopolymer polypropylene sample  
173 tube and filtered using FilterMate<sup>TM</sup>, 0.45  $\mu$ m PDVF cartridge with PTFE prefilter before being frozen  
174 on dry ice at the time of collection for long-term storage at  $-20^{\circ}$  C until ion chromatography (IC),  
175 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) and ammonia assays were  
176 carried out.

177 A Dionex ICS-1100 ion chromatography system was used to determine chloride ( $\text{Cl}^{-}$ ),  
178 phosphate ( $\text{PO}_4^{3-}$ ), sulphate ( $\text{SO}_4^{2-}$ ) and nitrate ( $\text{NO}_3^{-}$ ) concentration. Conditions were: column:  
179 Dionex IonPac AS14a (4mm\*250mm); delivery speed: 4 ml  $\text{min}^{-1}$ ; delay volume: 125  $\mu$ l; flow rate: 1  
180 ml  $\text{min}^{-1}$ ; eluents: 0.25 mM sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) and 0.25 mM sodium bicarbonate ( $\text{NaHCO}_3$ )  
181 in a ratio of 8:1. Total run time was 20 min. For metal concentrations, a Perkin Elmer Avio-500 ICP-

182 OES was used to determine Al, Ca<sup>2+</sup>, Cu, K<sup>+</sup>, Mg<sup>2+</sup>, Na<sup>+</sup> and Pb. A multi-element standard (Fisher  
183 Scientific, product code 1009-2633) was used for calibration. Filtered samples were acidified with  
184 nitric acid (1%) prior to analysis. The wavelengths used were: Al (396.153 nm), Ca<sup>2+</sup> (317 nm), Cu  
185 (324.752 nm), K<sup>+</sup> (766 nm), Mg<sup>2+</sup> (285 nm), Na<sup>+</sup> (589.592 nm), Pb (220.353 nm).

186 Total ammonia nitrogen levels were quantified using a microplate colourimetric procedure  
187 (Bower and Holm-Hansen, 1980). Standards were made by a dilution of 1 mM ammonium chloride  
188 (NH<sub>4</sub>Cl). Standards and samples were run in triplicate. The un-ionised ammonia (NH<sub>3</sub>) content was  
189 calculated from the total ammonia nitrogen (TAN) values, the pH and temperature (° C) (California  
190 Water Boards Agency, 2011).

### 191 2.3. Waterborne Pathogens

192 Water (50 ml) samples for microbiology analysis were collected in sterile falcon tubes and  
193 stored at 4° C until DNA extraction. Samples were centrifuged for 5 min at 5,000 g and filtered  
194 through a membrane filter (0.45 µm pore size). The Metagenomic DNA isolation kit for water  
195 (Epicentre®, an Illumina company) was used to isolate DNA from the sample water and the DNA  
196 pellet was stored at -20° C. Polymerase chain reaction (PCR) was carried out on the isolated DNA  
197 with genus- or species-specific primers (Table 1) to record the occurrence of nine pathogens, which  
198 were recorded as present or absent. Sterile PCR tubes were used to mix 24 µl of master mix and 1 µl  
199 of sample. A negative control was carried out for each gel containing the master mix and 1 µl of  
200 molecular water. The initial denaturation was 95° C for 2 min. Cycling conditions for all primers were  
201 35 cycles of 95° C for 30 s, primer-specific annealing temperature for 45 s and 72° C for 1 min. To  
202 ensure complete extension, the cycling conditions ended with a temperature rise to 72° C for 10 min.  
203 20 µl of each sample/negative control was analysed by gel electrophoresis on an ethidium bromide  
204 stained 2% agarose gel.

205 Table 1. Pathogens tested for presence/absence in the water and PCR master mix composition.

206

Primer	Forward Sequence (5'- 3')	Reverse Sequence (5'- 3')	Annealing
<i>Aeromonas</i> spp.	5'-TCCGTTGGATATGGCTCTTC-3'	5'-GCGTACCACGATGTCTGAA-3'	58° C
<i>Pseudomonas</i> spp.	5'-GACGGGTGAGTAATGCCTA-3'	5'-CACTGGTGTTCCCTCCTATA-3'	63° C
<i>Flavobacterium columnare</i>	5'-GCAGGATGACGGTCCTATGG-3'	5'-TTAACCTGACACCTCACGGC-3'	58° C
<i>Streptococcus</i> spp.	5'-GTACAGTTGCTTCAGGACGTATC-3'	5'-ACGTTTCGATTCATCACGTTG-3'	52° C
<i>Mycobacterium</i> spp.	5'- CCTTTCTAAGGAGCACACGA-3'	5'-TTGCTTAGATGCTCGCAACC-3'	58° C
<i>Escherichia coli</i>	5'-CGGACGGGTGAGTAATGTCT-3'	5'-GTTAGCCGGTGCTTCTTCTG-3'	65° C
<i>Acanthamoeba</i> spp.	5'-GGCCAGATCGTTTACCGTGAA-3'	5'-TCTCACAAGCTGCTAGGGGAGTCA-3'	62° C
<i>Vibrio anguillarum</i>	5'-AAGAAGCACCGGCTAACTCC-3'	5'-CCATGCAGCACCTGTCTTAC-3'	53° C
<i>Staphylococcus aureus</i>	5'-GCGATTGATGGTGATACGGTT-3'	5'-CAAGCCTTGACGAACTAAAGC-3'	55° C

## 207 2.4. Fish physiology and behaviour

208 A range of measures were taken to assess potential stress experienced by the fishes during  
209 transport and recovery. Physiological indicators of stress were studied by measuring levels of  
210 waterborne cortisol and ventilation rate. Behavioural signs of stress were recorded by observing  
211 stress-related behaviours displayed by the fish. Mortality levels were recorded daily by wholesaler  
212 and retail store staff. The physical condition of the fish was assessed by recording any visible injuries  
213 to both the body and fins using the video recordings collected for behavioural analysis. In addition,  
214 the eye colour of fish was calculated from video images to determine if it could be used as an  
215 indicator of stress, as has been done in other fish species (Cruz and Tauli, 2015; Freitas et al., 2014;  
216 Suter and Huntingford, 2002; Volpato et al., 2003).

### 217 2.4.1. *Waterborne Cortisol*

218 Water samples (100 ml) were collected in homopolymer polypropylene sample tubes, frozen  
219 immediately on dry ice and stored at -20° C until further analysis. Sep-Pak® Light C18 cartridges  
220 were primed with 1 ml methanol (100%) followed by 1 ml of double distilled water. Cortisol was  
221 extracted through the cartridges using a vacuum pump with a maximum flow rate of 1 ml min<sup>-1</sup>,  
222 followed by 1 ml of methanol (20%), then 1 ml of methanol (100%) to elute the cortisol. Methanol  
223 was evaporated using a nitrogen sample evaporator (Biotage®) at 45° C and reconstituted in 250 µl  
224 assay buffer. Cortisol concentrations were then measured using an enzyme-linked immunosorbent  
225 assay (ELISA) cortisol plate assay (Enzo Prod. No. ADI-900-071) with all samples and standards run  
226 in duplicate. Cortisol concentrations were adjusted for stocking density and duration of transport and  
227 expressed as cortisol released per fish per hour of transport (pg fish<sup>-1</sup> h<sup>-1</sup>).

### 228 2.4.2. *Behaviour*

229 Behavioural Observation Research Interactive Software (BORIS) (Friard and Gamba, 2016)  
230 was used to analyse behaviour displayed by the fish (Table 2). To determine reliability of analyses,  
231 10% of the video were analysed blind by a second observer and concordance was found to be high

232 between observers (Kendall's W coefficient: 0.96,  $p < 0.01$ ). Randomly selected individual fish were  
233 tracked for 3 min, and this was repeated consecutively from the start to the end of the video such that  
234 10 individuals were tracked. Displayed behaviours for each focal fish were totalled to obtain either a  
235 bag or tank score depending on the stage of transport. For neon tetras, only gasping, crowding and  
236 group formation were visible on the videos due to the small size of the fish. On arrival at the  
237 wholesaler while the fish were still in the bag, only gasping, crowding and group formation were  
238 measured due to visibility at high stocking densities. Only behaviours that were visible at specific  
239 stages of transport were included in statistical analyses.

240 Table 2. Ethogram of the behaviours recorded.

241

Behaviour	Description	Relation to welfare
Gasping	Approximate per cent of fish gasping at the surface of the water.	Gasping behaviour indicates low-quality water, specifically, low levels of DO (Kramer, 1987).
Crowding	Approximate per cent of fish grouped close (within 1 body length) together and swimming in the same direction.	Crowding increases with stress and can be a result of a perception of threat (Schreck et al., 1997).
Group formation	All fishes in the tank forming multiple schools with 30 cm between the groups.	The polarization of schools has been suggested to occur as a trade-off between predation risk and optimal foraging (Delgado De Carvalho et al., 2007).
Biting	Occurrence of fish biting or nipping a conspecific.	Biting can cause injuries and may lead to death. It is also a sign of aggression, or sub-optimal feeding (Håstein et al., 2005; Kalueff et al., 2013; Martins et al., 2012).
Erratic swimming	Occurrence of rapid swimming and direction change in the absence of being chased.	Erratic swimming is an indicator of elevated stress, distress or pathogenic condition and may be used as a sign of bad welfare (Conte, 2004; Håstein et al., 2005; White et al., 2017).
Latency to feed	Duration (s) from the time food is dropped in the tanks to the time 90% of the fish are feeding.	Latency to feed can indicate a reduced motivation to feed, an increased perception of risk to feed, reduced confidence to approach food (Magurran and Pitcher, 1983; Mikheev et al., 1994; Saxby et al., 2010).
Freezing	Duration (s) fish spend immobile.	Freezing is considered a fear and stress-related behaviour (Braithwaite and Salvanes, 2005; Koolhaas et al., 1999).
Ventilation rate	Measured by visually counting 20 successive opercular or buccal movements, measuring the elapsed time and then calculating the frequency per minute (based on Alvarenga and Volpato, 1995).	Ventilation rate can increase as a result of aquaculture procedures or stressors and is a highly sensitive mechanism involved in stress coping strategies (Barreto and Volpato, 2004; Martins et al., 2012).

### 2.4.3. *Mortality, Body Injuries and Eye Colour*

Any mortality was recorded daily by staff at the wholesaler and retail stores. From the recorded videos used for behavioural analysis, three still frames (one showing the full left flank, one showing the full right flank and one showing the tail) were taken to assess the physical condition of each of the 10 focal fish. Body injuries were recorded based on the following criteria: (0) no visual injuries; (1) single minor non-life-threatening injury; (2) multiple minor non-life-threatening injuries; (3) major life-threatening injury. Injuries to fins were recorded as follows: (0) no visible injury; (1); small section of fin missing; (2) large section of fin missing or shortening of the fin; (3) fin missing (Deng et al., 2005; Neitzel et al., 2004). The scores for fin injury and body injury were summed to obtain an overall injury score; these injuries were recorded for each fish and combined to obtain an injury score for all the fish transported together. Eye colour was measured only during recovery at the store because the methylene blue added to the water at the wholesaler affected the colouring of the water. Eye colour was assessed from one still frame per focal fish for oranda and variatus platy but not for neon tetra due to their small size. Photoshop Elements 15 was used to select the image of the iris. MatLab was used to determine the number of pixels on the grey scale pixel intensity scale (from 0-64 = black, 65-128 =dark grey, 129-192 = light grey, 193-255 = white). The percentage pixel of each intensity was calculated using the following equation:

$$\frac{\text{number of pixels at intensity}}{\text{total number of pixels}} \times 100$$

An average per cent of black and dark grey pixels was calculated for the 10 focal fish to obtain a tank average of eye darkening for each data collection point.

## 2.5. Ethical Approval

This study was approved by the University of the West of Scotland Ethics Committee as well as the Animal Welfare Ethics Review Board at the Waltham Petcare Science Institute. The samples collected in this study were collected non-invasively as part of routine transport of the fish for commercial purposes.

## 267 2.6. Statistical Analyses

268 Statistical analyses were carried out based on Bolker et al. (2009) using R ver. 3.6.1. (R Core  
269 Team, 2018). First, whether water quality parameters, behavioural and physiological measures and the  
270 percentage of water samples containing each pathogen differed between each stage of transport was  
271 considered. Error structures including homogeneity of variances for linear and generalized linear  
272 models were determined by visual inspection of residuals and QQ plots (R Core Team, 2018). The  
273 normally distributed response variables were fitted to a general linear mixed model with stage of  
274 transport (i.e. arrival at wholesaler, day 1 at wholesaler etc.) and species as fixed factors, and with  
275 sampling location (i.e. wholesaler and specific stores) and bag number (out of 10 replicate blocks for  
276 each species) as random terms using the lme function from the nlme package (Pinheiro et al., 2018).  
277 For non-normally distributed response variables, the log and square root functions were applied. The  
278 transformation was tested by checking the distribution of the residuals as described above and the best  
279 transformation was selected. If the data could not be transformed to normality, a generalised linear  
280 mixed model was carried out using Poisson family from the lme4 package (Bates et al., 2015) with  
281 random explanatory variables designated as before and the data were checked for over-dispersion  
282 using the blmecco package (Korner-Nievergelt et al., 2015). Fixed explanatory variables that did not  
283 significantly improve the fit of the model based on delta AIC in stepAIC were removed from the  
284 model (Venables and Ripley, 2002). Percentage data were expressed as proportions and a binomial  
285 generalised linear mixed model (GLMM) was carried out with the fixed and random variables as  
286 described above. Model simplification was performed using delta AIC as previously described and P  
287 values for fixed terms derived from Chi-square log-likelihood test. The significance of the covariates  
288 was tested by ANOVA (using the Anova function in the car package) (Fox and Weisberg, 2011;  
289 Pinheiro et al., 2018). Due to the large number of comparisons, a Bonferroni correction was carried  
290 out using the emmeans package (Lenth et al., 2019). The figures were created using the ggplot2  
291 package (Wickham, 2016).

292 The effect of pathogen presence on behaviour and physiological responses was determined by  
293 model analysis. For each response variable a general or generalised linear model was carried out as



294 described above with presence of each pathogen set as explanatory variables. Model selection *via* AIC  
 295 was carried out as described above to identify pathogens that significantly affected the response  
 296 variable. T-tests were carried out for each selected pathogen to determine the effect of pathogen  
 297 presence on response behaviour.

298 Following separate analyses into changes in water quality and welfare between the stages of  
 299 transport, the influence of water quality on welfare (i.e. behaviour and physiology measures) was  
 300 considered. To do this, distance-based linear models (DistLM) were used to determine if water  
 301 quality influenced behaviour. DistLM analyses were carried out using PRIMER 7 software (Clarke  
 302 and Gorley, 2015) with the PERMANOVA+ add-on (Anderson, Gorley and Clarke, 2016). The  
 303 chemistry data were standardised to a mean of 0 and standard deviation of 1, and a resemblance  
 304 analysis was carried out for the chemistry and behavioural data to measure distance using Euclidean  
 305 distance. DistLM was carried out step-wise to select for the optimum model based on the AIC.

306

### 307 **3. Results**

#### 308 **3.1. Water Chemistry**

309 The water chemistry parameters measured were each analysed for differences between species and  
 310 between each stage of the transport chain. DO was significantly affected by both species and stage of  
 311 transport (Fig. 1A; Table 3).

312 Table 3. ANOVA result for each recorded water quality parameter for stage of transport, species and  
 313 in the interaction between stage of transport and species showing F values, degrees of freedom (df),  
 314 and p values.

	Transport Stage	Species	Transport Stage*Species
DO	F <sub>8,218</sub> =132.43, p<0.001	F <sub>8,218</sub> =12.69, p<0.001	F <sub>16,218</sub> =4.88, p<0.001
Temperature	F <sub>8,220</sub> =11.5, p<0.001	F <sub>2,220</sub> =42.3, p<0.001	F <sub>16,220</sub> =9.3, p<0.001
pH	F <sub>8,220</sub> =11.175, p<0.001	F <sub>2,220</sub> =7.78, p=0.001	F <sub>16,220</sub> =1.43, p=0.214

KH	$F_{8,238}=3.58, p=0.002$	$F_{2,236}=0.503, p=0.781$	$F_{61,220}=0.378, p=0.998$
TAN	$F_{8,220}=23.64, p<0.001$	$F_{2,220}=9.658, p<0.001$	$F_{16,220}=4.557, p<0.001$
NH <sub>3</sub>	$F_{8,238}=2.59, p=0.012$	$F_{2,236}=0.245, p=0.874$	$F_{16,220}=2.3215, p=0.008$
NO <sub>3</sub> <sup>-</sup>	$F_{8,235}=25.226, p<0.001$	$F_{2,235}=3.417, p=0.059$	$F_{16,219}=1.106, p=0.543$
SO <sub>4</sub> <sup>2-</sup>	$F_{8,219}=11.076, p<0.001$	$F_{2,219}=4.376, p=0.025$	$F_{16,219}=4.153, p<0.001$
Cl <sup>-</sup>	$F_{8,238}=20.49, p<0.01$	$F_{2,236}=0.841, p=0.571$	$F_{16,202}=2.691, p=0.018$
PO <sub>4</sub> <sup>3-</sup>	$F_{8,236}=4.73, p<0.01$	$F_{2,234}=1.448, p=0.237$	$F_{16,218}=0.705, p=0.879$
Na <sup>+</sup>	$F_{8,236}=7.21, p<0.01$	$F_{2,236}=3.34, p=0.071$	$F_{16,220}=0.472, p=0.998$
Ca <sup>2+</sup>	$F_{8,263}=4.421, p<0.001$	$F_{2,236}=4.228, p=0.035$	$F_{16,220}=0.267, p=0.998$
K <sup>+</sup>	$F_{8,220}=0.697, p=0.83$	$F_{2,220}=5.29, p=0.012$	$F_{16,220}=0.539, p=0.998$
Mg <sup>2+</sup>	$F_{8,220}=0.63, p=0.875$	$F_{8,244}=9.46, p<0.01$	$F_{16,220}=0.811, p=0.826$

315

316 DO was higher on arrival at both the wholesaler and the retail store and was higher in the neon  
317 tetra water on arrival at the wholesaler than in the oranda and variatus platy water. DO remained  
318 higher in the neon tetra tanks than in the oranda tanks on day 1 and 2 of recovery at the wholesaler  
319 (Fig. 1A). Water temperature was affected by transport stage (Table 3) and species. The water  
320 temperature experienced by neon tetras did not change throughout their transport, whereas both  
321 orandas and variatus platy experienced a decrease in temperature at the retail stores (Fig. 1B). For all  
322 species, water pH was significantly lower on arrival at the wholesaler than at subsequent transport  
323 stages and throughout the pH in the neon tetra water was higher than that of oranda water (Fig. 2A).  
324 KH was significantly higher on arrival at the wholesaler than in the water fish were packed in for  
325 transport to the retail stores (Fig. 2B; Table 3). Species and the interaction between species and  
326 transport stage was not significant (Table 3).

327 TAN was significantly affected by stage of transport and species and the two factors interacted  
328 significantly (Fig. 3A; Table 3). TAN water levels on arrival at the wholesaler were significantly  
329 higher in neon tetra and oranda bags than during the subsequent transport stages (Fig. 3A). On day 1  
330 of recovery at the store, TAN was higher in variatus platy tanks than in neon tetra tanks; on day 2 of  
331 recovery at the store, TAN was higher in oranda tanks than in neon tetra tanks (Fig. 3A). NH<sub>3</sub> was  
332 lower during recovery at the wholesaler than during the other transport stages with inter-specific  
333 differences in NH<sub>3</sub> dependent upon transport stage (Fig. 3B; Table 3).

334 There was a significant effect of transport stage on NO<sub>3</sub><sup>-</sup> and the effect of species approached  
335 significance (Fig. 4A; Table 3). For all three species, NO<sub>3</sub><sup>-</sup> concentrations were significantly higher in  
336 the tank water during recovery at the stores than during the previous transport stages. SO<sub>4</sub><sup>2-</sup>  
337 concentrations were affected by the stage of transport and species (Fig. 4B; Table 3). SO<sub>4</sub><sup>2-</sup>  
338 concentrations in the water of neon tetra bags were significantly lower on arrival at the retail stores  
339 than in the tanks during recovery at the stores. Variatus platy were exposed to significantly higher  
340 SO<sub>4</sub><sup>2-</sup> concentrations in bags on arrival at the wholesaler than in the tanks during recovery at the  
341 wholesaler and packing for shipment to the stores (Fig. 4B). For all three species, Cl<sup>-</sup> concentrations  
342 were lower in tanks during recovery at the retail store than at the wholesaler (Fig. 4C; Table 3). PO<sub>4</sub><sup>3-</sup>  
343 concentrations were significantly lower in bag water on arrival at the stores than in tanks during  
344 recovery at the stores (Fig. 4D; Table 3). For all three species, water Na<sup>+</sup> concentration was  
345 significantly higher on arrival at the retail store than during recovery at the stores (Fig. 5A; Table 3)  
346 and water Ca<sup>2+</sup> concentrations were significantly lower on arrival at the retail store than during  
347 recovery at the store (Fig. 5B). Concentrations of K<sup>+</sup> were lower in the water of neon tetra than in the  
348 water of variatus platy but were not different to the water for oranda (Fig. 5C; Table 3) and Mg<sup>2+</sup>  
349 levels were higher in variatus platy water than in the neon tetra water or the oranda water (Fig. 5D;  
350 Table 3). Al, Cu and Pb were below detection limits (<0.008 ppm).

351

## 352 3.2. Waterborne Pathogens

353 The presence/absence of pathogens in water samples taken at each stage of transport was analysed  
354 across all species of fish. The percentage of samples that contained *Aeromonas* spp. was significantly  
355 higher on arrival at the wholesaler than in the water samples when fish were packed for transport to  
356 retail stores and on day 5 of recovery at the store ( $X^2=31.093$ ,  $df=2$ ,  $p<0.0001$ ; Fig. 6A). *Pseudomonas*  
357 spp. was present in significantly more samples on arrival at the wholesaler than at packing for  
358 transport to retail stores and the number of positive samples was significantly lower again on day 5 of  
359 recovery at the stores ( $X^2=30.094$ ,  $df=2$ ,  $p<0.0001$ ; Fig. 6B). *Mycobacterium* spp. presence  
360 significantly decreased between arrival at the wholesaler and day 5 post-transport at the retail store  
361 ( $X^2=55.735$ ,  $df=2$ ,  $p<0.0001$ ; Fig. 6C). *Mycobacterium* spp. was present in every sample at packing  
362 for transport to retail stores, this caused a lack of variation and post-hoc testing for this data point was  
363 not possible. The number of positive samples for *Acanthamoeba* spp. ( $21.59\% \pm 41.38$  SD), *E. coli*  
364 ( $97.73\% \pm 14.99$  SD), *F. columnare* ( $95.45\% \pm 20.94$  SD), *Streptococcus* spp. ( $54.54\% \pm 50.01$  SD),  
365 *S. aureus* ( $21.59\% \pm 41.38$  SD) and *V. anguillarum* ( $98.86\% \pm 10.66$  SD) did not change significantly  
366 through the three tested stages of transport.

### 367 3.3. Fish physiology and behaviour

368 In neon tetras only two behaviours showed significant changes through transport stage. Group  
369 formation behaviour was greater on day two of recovery at the wholesaler than at the other stages of  
370 transport, except for day 1 of recovery at the wholesaler and at the store ( $F_{8,60}=4.711$ ,  $p=0.004$ ; Fig.  
371 7A). Latency to feed was significantly lower during recovery at the retail stores than during recovery  
372 at the wholesaler ( $F_{3,33}=19.404$ ,  $p<0.001$ ; Fig. 7B). In oranda, the occurrence of erratic swimming was  
373 highest on day 5 of recovery at the store ( $F_{1,8}=5.66$ ,  $p=0.003$ ; Fig. 8). In variatus platy, occurrence of  
374 erratic swimming significantly decreased between day 1 of recovery at the wholesaler and day 2 and 5  
375 of recovery at the wholesaler (Fig. 9A). Variatus platy displayed significantly less erratic swimming  
376 during recovery at the store than at the wholesaler ( $F_{6,38}=10.269$ ,  $p<0.001$ ; Fig. 9A). Biting  
377 behaviour was significantly affected by the transport stage with a general increase seen in the  
378 occurrence of biting behaviour through transport stages and the occurrence of biting was significantly  
379 higher at day 5 of recovery at the store than the previous stages ( $F_{6,38}=7.618$ ,  $p<0.001$ ; Fig. 9B). The

380 occurrence of freezing behaviour was significantly higher on day 1 of recovery at the store than all the  
381 other recorded stages of transport, except for day 2 of recovery at the store ( $F_{6,38}=4.569$ ,  $p=0.002$ ; Fig.  
382 9C). Erratic swimming, biting and freezing behaviour could not be observed on arrival at the  
383 wholesaler or the store, therefore these time points were excluded from the analysis. No other  
384 significant changes were recorded in behavioural and physiological measures (Supplementary  
385 Material, Table 1). In orandas, the presence of *Mycobacteria* spp. in the water resulted in a  
386 significantly darker eye colour ( $t\text{-test}=2.611$ ,  $p=0.026$ ; Fig. 10). No other pathogen was found to have  
387 a significant effect on any of the recorded behaviour and health measures ( $p<0.05$ ).

388 Distance-based linear models revealed that, in neon tetra, 56.03% of variation in the latency to  
389 feed was associated with  $\text{NO}_3^-$ ,  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{PO}_4^{3-}$  and  $\text{Mg}^{2+}$  concentrations (Table 4) changes in group  
390 formation were not attributable to water chemistry. Copper and TAN explained 32.19% of the  
391 variation in erratic swimming behaviour in oranda whereas in variatus platy, 62.52% of variation in  
392 erratic swimming was explained by DO,  $\text{Cl}^-$ ,  $\text{NO}_3^-$  and  $\text{K}^+$  (Table 3). Variation in biting behaviour  
393 (35.26%) displayed by variatus platy was explained by temperature, pH and  $\text{NH}_3$  and variation in  
394 freezing behaviour (18.15%) was explained by  $\text{NO}_3^-$  and pH (Table 4).

395

396 Table 4. Results for behavioural variation in neon tetra, oranda and variatus platy showing the  
 397 chemical parameters selected via DistLM indicating Pseudo-F, p value and residual degrees of  
 398 freedom (df).

Species	Behaviour	DistLM	Contribution (%)	Pseudo-F	p value	Residual df
Neon tetra						
	Latency to feed					
		NO <sub>3</sub> <sup>-</sup>	28.625	22.459	0.001	56
		Cl <sup>-</sup>	14.899	14.510	0.001	55
		Na <sup>+</sup>	5.515	5.843	0.013	54
		PO <sub>4</sub> <sup>3-</sup>	3.585	4.010	0.044	53
		Mg <sup>2+</sup>	3.413	4.037	0.056	52
Oranda						
	Erratic swimming					
		Cu	29.323	33.191	0.005	80
		TAN	2.872	3.346	0.059	79
Variatus platy						
	Erratic swimming					
		DO	41.493	41.843	0.001	59
		Cl <sup>-</sup>	16.240	22.285	0.001	58
		NO <sub>3</sub> <sup>-</sup>	2.508	3.596	0.062	57
		K <sup>+</sup>	2.283	3.412	0.071	56
	Biting					
		temperature	16.965	12.462	0.002	61
		pH	13.253	11.396	0.004	60
		NH <sub>3</sub>	2.779	2.447	0.116	59
		Cl <sup>-</sup>	2.262	2.026	0.133	58
	Freezing					
		NO <sub>3</sub> <sup>-</sup>	9.699	6.444	0.026	60
		pH	8.454	6.094	0.022	59

## 400 **4. Discussion**

401 The aim of this study was to record a range of water quality and health (physiological and  
402 behavioural) parameters throughout the stages of commercial transport for neon tetra, oranda and  
403 variatus platy. This is the first study to our knowledge that has collected these measures through  
404 multiple stages of commercial transport. Overall, we found that water quality fluctuated greatly, albeit  
405 within safe levels for the fishes, throughout the stages of the recorded supply chain; all of the tested  
406 pathogens were present within this supply chain at different levels. The fluctuation in water chemistry  
407 parameters recorded in this study could be a source of stress for the fishes within the ornamental trade  
408 that is rarely if ever considered. Out of the recorded stress measures, behaviour was found to be the  
409 most sensitive, changing the most across stages of transport. Behavioural changes have never been  
410 tested as an indicator of stress in ornamental fish studies which often employ invasive or lethal  
411 methodologies.

412 Water quality was high from arrival at the wholesaler to recovery at the stores. Throughout the  
413 stages of transport, oxygen was on average above 100% saturation. The mean water pH was 6.7 with  
414 a few instances where pH dropped below 5.5. The range in water pH between stores was large due to  
415 the geographical distribution of the stores. Small daily fluctuations in pH are unlikely to have a long  
416 term effect on fish, although this will be species-dependent (Cecil, 1999; Oliveira et al., 2008; Roberts  
417 and Palmeiro, 2008). High levels of  $\text{NH}_3$  are toxic to fishes and can lead to mortality (LC50 toxicity  
418  $\sim 2.79 \text{ mg l}^{-1}$  in freshwater fishes at pH 8 and  $20^\circ\text{C}$  based on 32 freshwater species but LC50 is  
419 different between species) (Randall and Tsui, 2002, USEPA, 1984). Average levels of  $\text{NH}_3$  were  
420  $0.003 \text{ mg l}^{-1}$  and any peaks in  $\text{NH}_3$  were still well below toxic levels (recorded peaks  $< 0.1 \text{ mg l}^{-1}$ ).  
421 Liquid zeolite (25 ml) was used during the international leg of the transport chain to regulate  
422 ammonia concentrations. The ammonia concentrations in the present study ( $0.005 \text{ mg l}^{-1}$ ) are in line  
423 with the zeolite treatment in a study by Bower and Turner (1982) ( $0.007 \text{ mg l}^{-1}$ ) who compared  $\text{NH}_3$   
424 levels in bags containing 0-20 g zeolites following a 24 h simulated transport with 10 or 20 goldfish  
425 (*C. auratus*) in 500 ml water. Elevated  $\text{NO}_3^-$  levels ( $> 50 \text{ mg l}^{-1}$ ) can lead to poor growth, lethargy,

426 anorexia and opportunistic infections (Roberts and Palmeiro, 2008). The levels recorded in this study  
427 were on average  $<17 \text{ ml l}^{-1}$ , well below this threshold.

428 The present study measured a range of chemical elements including  $\text{NO}_3^-$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$  and trace  
429 metals. The results showed that the levels of some chemical elements in the water fluctuated between  
430 species (potassium and magnesium), between stages of transport ( $\text{PO}_4^{3-}$  and sodium) or between both  
431 species and stages ( $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{Cl}^-$  and  $\text{Ca}^{2+}$ ). Similar data were collected in a previous study which  
432 compared ion levels in water between multiple water sources of a commercial supply chain in Nile  
433 tilapia (*Oreochromis niloticu*) (Colt et al., 2011). This fluctuation in chemical elements highlights that  
434 ornamental fishes can be exposed to different water qualities through the supply chain. Fluctuation in  
435 water chemistry between times points is likely due to daily or geographical fluctuations but further  
436 research is needed. It is unclear why water chemistry was different between species within a time  
437 point as they were always exposed to water from the same source. Differences in feed composition or  
438 fish physiology might result in the observed fluctuation however this requires further investigation. In  
439 this study, the trace metals measured were below the detection limit for the majority of samples; the  
440 few samples that did contain detectable levels were within levels considered safe for freshwater fishes  
441 (Poléo, 1995; Roberts and Palmeiro, 2008). Overall whilst water quality was appropriate for each  
442 species at all stages of transport, fishes experienced significant changes in water chemistry between  
443 different stages; the effect of this fluctuation in water quality demands further examination.

444 The water was tested for the presence of common bacterial pathogens and a free-living amoeba  
445 which can harbour pathogens. None of the water samples collected were pathogen-free with *E. coli*, *F.*  
446 *columnare* and *V. anguillarum* pathogens being found in over 90% of the samples. The number of  
447 samples which tested positive for *Aeromonas* spp., *Pseudomonas* spp. and *Mycobacterium* spp. was  
448 significantly lower on day 5 at the retail stores than on arrival at the wholesaler. However, there was  
449 no clear pattern in the presence/absence of the other pathogens across the transport chain indicating  
450 that the microbial control measures taken by the wholesaler (tetracycline) and the stores (UV light  
451 filter) reduce some of the pathogenic microbial load in the supply chain. Rose et al. (2013) found  
452 bacteria isolated from 15 genera in imported ornamental fishes and associated water, however their



453 detection of common fish pathogens was low for some genera (e.g. *Vibrio* spp.). Other studies, such  
454 as Trust and Bartlett (1974) and Zanoni et al. (2008), also detected pathogenic genera (e.g. *Vibrio* spp.  
455 and *Mycobacterium* spp.) in ornamental fishes or their water that were either recently imported or sold  
456 in retail stores. The difference in detected genera between studies shows how variable the bacterial  
457 load can be in supply chains. Many importers receive fishes from several farms and continents; while  
458 at the importer, cross-contamination can occur between species from different origins *via* husbandry  
459 practices resulting in the introduction of potential pathogens in non-contaminated water. Further  
460 research is needed to better understand the prevalence and transfer of pathogens into the ornamental  
461 fish trade.

462 In the present study, only microbial presence in the water was tested. Although no disease  
463 outbreaks were recorded during this study, it was not possible to determine whether all the fish were  
464 disease-free. In oranda, eye colour was significantly darker in the presence of *Mycobacterium* spp.,  
465 however, there were no other typical signs of mycobacteriosis seen (scale loss, anorexia, lethargy,  
466 skin ulcers, fin and tail rot) (Alapide-Tendencia and Peña, 2002; Austin and Austin, 2012; Smith,  
467 1997). Over 150 species make up the genus *Mycobacterium* spp. and at least 20 species have been  
468 associated with fish, however, it is not always clear if species are the causative agent of  
469 mycobacteriosis as multiple species have been detected in diseased fish (Keller et al., 2018). As the  
470 primers used to test for *Mycobacterium* in the present study were genus specific and not species-  
471 specific, it cannot be determined if the fish were at risk of mycobacteriosis. *Mycobacterium* spp. can  
472 cause both depigmentation or hyperpigmentation of fishes (Smith, 1997) but the author does not  
473 specify which species causes this colour change. The identity of species causing and whether  
474 colouration of the eye is affected by *Mycobacterium* spp. in the same way as body colour is unknown.  
475 Eye colour in fishes has also been reported to darken with stress including social stress (Suter and  
476 Huntingford, 2002; Freitas et al., 2014; Volpato et al., 2003). Further research is needed to better  
477 understand causes and correlates of eye colour changes in fishes.

478 Fishes can become susceptible to many pathogens during periods of high stress as a result of a  
479 weakened immune system (Scholz, 1999; Yanong, 2010). It is therefore important to recognise signs

480 of stress and poor welfare in ornamental fishes during commercial transport which may indicate  
481 increased susceptibility to waterborne pathogens. Mortality levels in our study were low for all three  
482 species and were not affected by transport stage. Other studies suggest that mortality is likely to be  
483 higher during recovery periods than immediately post-transport (Ali et al., 1989; Kilgore et al., 2009)  
484 so it may be of benefit in the future to monitor mortality at retail stores over a longer time period. Of  
485 course, retail stores are usually not the end-point of the journey of an ornamental fish and  
486 understanding mortality effects may need to include the journey from retailer to owner and recovery  
487 in home aquaria.

488 Out of the recorded stress measures, behaviour was found to change the most between stages of  
489 transport. Due to the small size of neon tetra, observing individual's behaviour was not possible,  
490 therefore, studying behaviour of this species in a commercial setting is challenging. Oranda had low  
491 levels of activity at most stages of the transport chain although a small increase in erratic swimming  
492 was seen during recovery at the stores. This increase in erratic swimming was small, however and  
493 may not represent a biologically relevant change. In contrast, *variatus platy* displayed the greatest  
494 changes in behaviours across the transport chain. The high levels of erratic swimming at the  
495 wholesaler may be associated with the activity of wholesaler staff, which were observed to disturb the  
496 fish. Housing *variatus platy* in tanks in a quieter area of the wholesaler facilities could therefore help  
497 reduce stress for this species. At the retail stores, erratic swimming was much lower in *variatus platy*  
498 compared to at the wholesalers which could be due to differences in housing conditions (e.g. stocking  
499 density, lighting, enrichment). In contrast, biting increased during recovery at the stores. Biting occurs  
500 in particular during the establishment of hierarchies and can result in stress and injury (Noble et al.,  
501 2012; Pitcher, 1986). This increase in biting behaviour in *variatus platy* at the retail stores was  
502 accompanied by a decrease in freezing behaviour, likely as a result of the fish avoiding bites and  
503 aggression from conspecifics (Braddock, 1945; Scott and Currie, 1980). The decrease in erratic  
504 swimming behaviour from the wholesaler to the store may indicate a reduction in stress following  
505 transport. It is possible that *platys* were still experiencing transport-related stress during recovery at  
506 the wholesaler. As this stress started to reduce during recovery at the stores, establishment of

507 hierarchies may have been initiated indicated by the progressive increase in biting behaviour  
508 (Braddock, 1945; Scott and Currie, 1980; Noble et al., 2012). Continued aggression beyond the initial  
509 hierarchy establishment could be detrimental causing chronic stress in some individuals (Sloman and  
510 Armstrong, 2002), therefore practices promoting more rapid and peaceful hierarchy establishment  
511 would improve the welfare of ornamental fish following transport.

512 Behavioural changes were found to be species-specific, therefore study species must be selected  
513 with this in mind, and a species-specific ethogram is needed when using changes in behaviour as an  
514 indicator of stress. Our multivariate analyses indicate that some fluctuation in water chemistry can  
515 influence stress-associated behaviours. Studies focusing on the effect of low water quality in  
516 ornamental fish welfare tend to focus on mortality or physiological response (Gonzalez et al., 1998;  
517 Brinn et al., 2012), however, here we show that water quality can also influence the behaviour of  
518 fishes. Future studies on the response of fishes to water quality should record behavioural changes as  
519 this may provide additional information (Abreu et al., 2014; Brinn et al., 2012). Other stress measures  
520 tested in this study did not vary between transport stages. Although large concentrations of water-  
521 borne cortisol were measured in the water on arrival at the wholesaler and arrival at the stores, when  
522 adjusted to account for excretion per fish per hour, the levels between the two data collection points  
523 were not significantly different. Eye darkening was not found to vary across the transport chain  
524 however, eye colour measurement was limited by the use of methylene blue and the variability in  
525 lighting conditions at the wholesaler. Eye darkening as a stress-indicator in a brighter, more controlled  
526 environment might be more reliable.

## 527 Conclusion

528 This study is the first to record water quality and ornamental fish welfare through a commercial  
529 chain. This study emphasises the complexity of studying ornamental fish welfare during commercial  
530 transport and that exposure to changes in water chemistry, within acceptable water quality parameters,  
531 should also be considered in studies of fish transport as water quality was found to influence the  
532 recorded behaviours. The study of animals within commercial transport chains often requires non-

533 invasive methods; here we find that monitoring behaviour is a useful tool but one that is species-  
534 specific.

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848

## 849 **6. Declaration of interest**

850 One author was employed by Pets at Home, he was not involved in data collection or analysis but was  
851 involved in the experimental design and provided feedback on earlier drafts of the manuscript.

852



854 **Figure Legends**

855 Figure 1 (A) DO levels (%) (n=10) and (B) temperature (° C) (n=10) through the transport stages (WS  
 856 A=wholesaler arrival, WS 1= wholesaler recovery day 1, WS 2= wholesaler recovery day 2, WS 5=  
 857 wholesaler recovery day 5, P= packing for transport to stores, Store A= store arrival, Store 1= store  
 858 recovery day 1, Store 2= store recovery day 2, Store 5= store recovery day 5) showing the mean  
 859 (diamond), median, upper and lower 25th percentile, and outliers. Letters indicate differences between  
 860 species within a specific time point where boxes sharing a letter are not significantly different.  
 861 Asterisks indicate significant differences within a species between different transport stages (post-  
 862 hoc Tukey, P<0.05).

863 Figure 2: (A) pH (n=10) through the transport stages (WS A=wholesaler arrival, WS 1= wholesaler  
 864 recovery day 1, WS 2= wholesaler recovery day 2, WS 5= wholesaler recovery day 5, P= packing for  
 865 transport to stores, Store A= store arrival, Store 1= store recovery day 1, Store 2= store recovery day  
 866 2, Store 5= store recovery day 5) showing the mean (diamond), median, upper and lower 25th  
 867 percentile, and outliers. Asterisks indicate significant differences within a species between different  
 868 transport stages. (B) Kh (° dKh) showing mean and standard error (there was no significant difference  
 869 between species (see text) so the data were combined across species, n=30). Letters indicate  
 870 differences between time points where boxes sharing a letter are not significantly different (post-hoc  
 871 Tukey, P<0.05).

872 Figure 3: (A) TAN (ppm) (n=10) and (B) NH<sub>3</sub> (n=10) through the transport stages (WS A=wholesaler  
 873 arrival, WS 1= wholesaler recovery day 1, WS 2= wholesaler recovery day 2, WS 5= wholesaler  
 874 recovery day 5, P= packing for transport to stores, Store A= store arrival, Store 1= store recovery day  
 875 1, Store 2= store recovery day 2, Store 5= store recovery day 5) showing the mean (diamond),  
 876 median, upper and lower 25th percentile, and outliers. Letters indicate differences between species  
 877 within a specific time point where boxes sharing a letter are not significantly different. Asterisks  
 878 indicate significant differences within a species between different transport stages (post-hoc Tukey,  
 879 P<0.05).

880 Figure 4: (A) NO<sub>3</sub><sup>-</sup> (n=10), (B) SO<sub>4</sub><sup>2-</sup> (n=10), (C) Cl<sup>-</sup> (n=10) and (D) PO<sub>4</sub><sup>3-</sup> (n=30) through the  
 881 transport stages (WS A=wholesaler arrival, WS 1= wholesaler recovery day 1, WS 2= wholesaler  
 882 recovery day 2, WS 5= wholesaler recovery day 5, P= packing for transport to stores, Store A= store  
 883 arrival, Store 1= store recovery day 1, Store 2= store recovery day 2, Store 5= store recovery day 5)  
 884 showing the mean (diamond), median, upper and lower 25th percentile, and outliers. (A-C) Letters  
 885 indicate differences between species within a specific time point where boxes sharing a letter are not  
 886 significantly different. Asterisks indicate significant differences within a species between different  
 887 transport stages. (D) Letters indicate differences between time points where boxes sharing a letter are  
 888 not significantly different. (post-hoc Tukey, P<0.05).

889 Figure 5: (A) Na<sup>+</sup> (n=30), (B) Ca<sup>2+</sup> (n=10), (C) K<sup>+</sup> (n=10) and (D) Mg<sup>2+</sup> (n=10) through the transport  
 890 stages (WS A=wholesaler arrival, WS 1= wholesaler recovery day 1, WS 2= wholesaler recovery day  
 891 2, WS 5= wholesaler recovery day 5, P= packing for transport to stores, Store A= store arrival, Store  
 892 1= store recovery day 1, Store 2= store recovery day 2, Store 5= store recovery day 5) and species  
 893 showing the mean (diamond), median, upper and lower 25th percentile, and outliers. (A) Letters  
 894 indicate differences between time points where boxes sharing a letter are not significantly different.  
 895 (B) Asterisks indicate significant difference within a species between different transport stages. (C-D)  
 896 Letters indicate differences between species (post-hoc Tukey, P<0.05).

897 Figure 6: Presence of (A) *Aeromonas* sp. (%), (B) *Pseudomonas* sp. (%), (C) *Mycobacterium* sp. (%)  
 898 through the transport, n=30. Letters indicate significant difference between transport stages where  
 899 bars sharing a letter are not significantly different (post-hoc Tukey, P<0.05).

900 Figure 7: Neon tetra behaviour, (A) crowding behaviour (%), (B) occurrence of group formation and  
901 (WS A=wholesaler arrival, WS 1= wholesaler recovery day 1, WS 2= wholesaler recovery day 2, WS  
902 5= wholesaler recovery day 5, P= packing for transport to stores, Store A= store arrival, Store 1= store  
903 recovery day 1, Store 2= store recovery day 2, Store 5= store recovery day 5) showing the mean  
904 (diamond), median, upper and lower 25th percentile, and outliers, n=10. Letters indicate differences  
905 between time points where boxes sharing a letter are not significantly different (post-hoc Tukey,  
906  $P<0.05$ ).

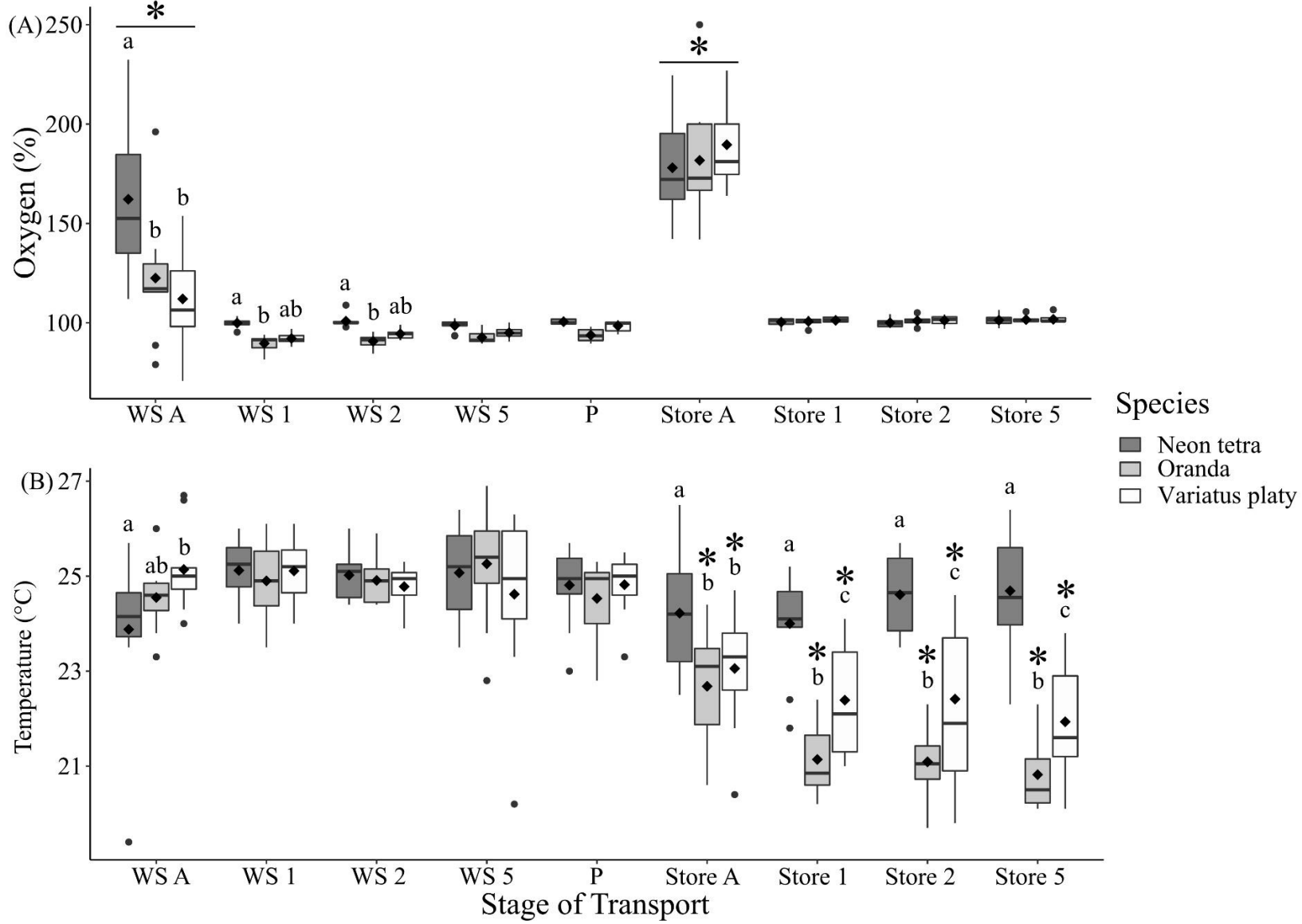
907 Figure 8: Erratic swimming behaviour in oranda through the transport stages (WS A=wholesaler  
908 arrival, WS 1= wholesaler recovery day 1, WS 2= wholesaler recovery day 2, WS 5= wholesaler  
909 recovery day 5, P= packing for transport to stores, Store A= store arrival, Store 1= store recovery day  
910 1, Store 2= store recovery day 2, Store 5= store recovery day 5) showing the mean (diamond),  
911 median, upper and lower 25th percentile, and outliers, n=10. Letters indicate differences between time  
912 points where boxes sharing a letter are not significantly different (post-hoc Tukey,  $P<0.05$ ).

913 Figure 9: Variatus platy behaviour, occurrence of (A) erratic swimming, (B) biting and (C) freezing  
914 through the transport stages (WS 1= wholesaler recovery day 1, WS 2= wholesaler recovery day 2,  
915 WS 5= wholesaler recovery day 5, P= packing for transport to stores, Store 1= store recovery day 1,  
916 Store 2= store recovery day 2, Store 5= store recovery day 5) showing the mean (diamond), median,  
917 upper and lower 25th percentile, and outliers, n=10. Letters indicate differences between time points  
918 where boxes sharing a letter are not significantly different (post-hoc Tukey,  $P<0.05$ ).

919 Figure 10: Oranda eye colour by the presence of *Mycobacterium* sp. showing the mean (diamond),  
920 median, upper and lower 25th percentile, and outliers, n=10. Asterisks indicate difference (post-hoc  
921 Tukey,  $P<0.05$ ).

922

Figure 1



923

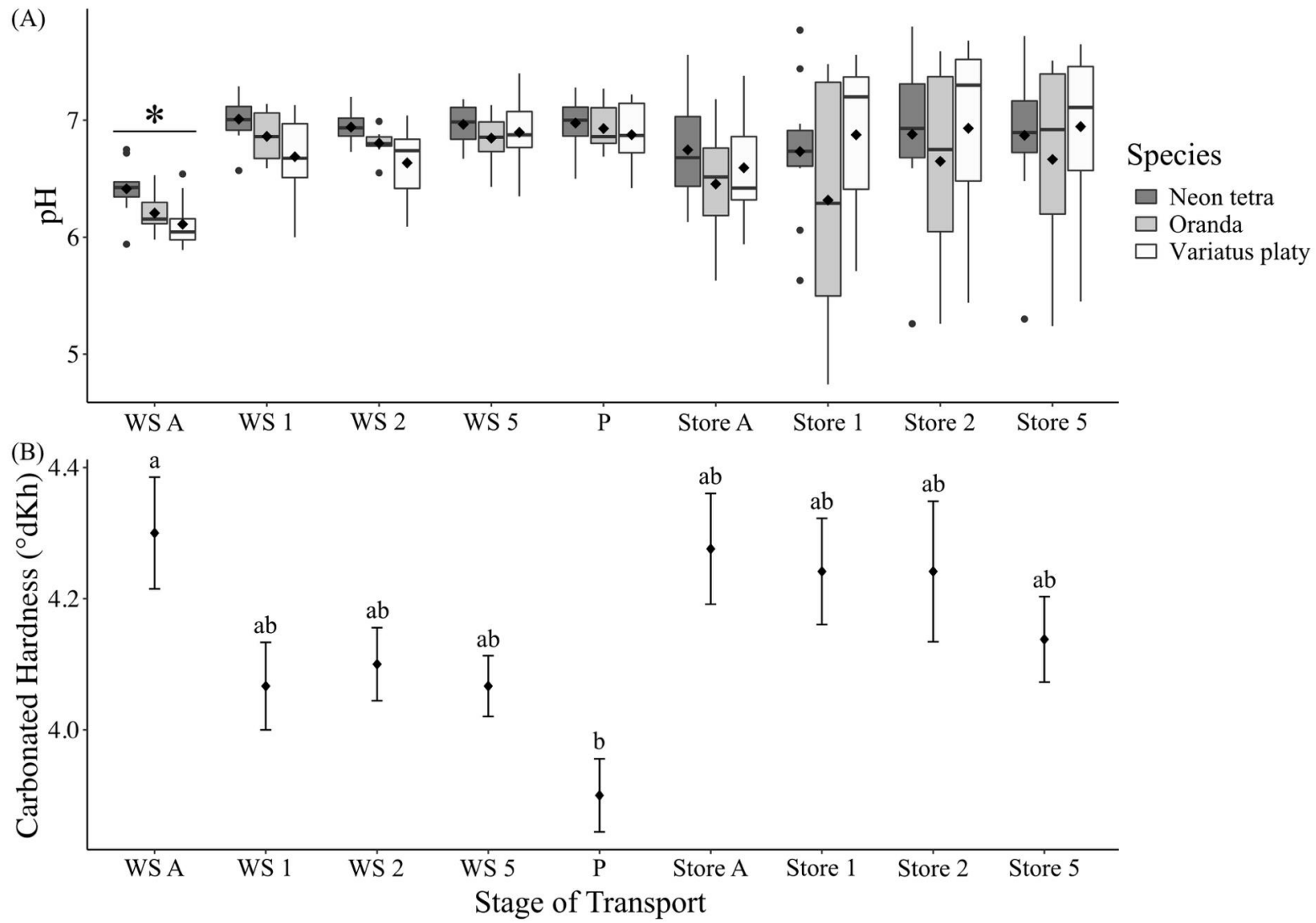
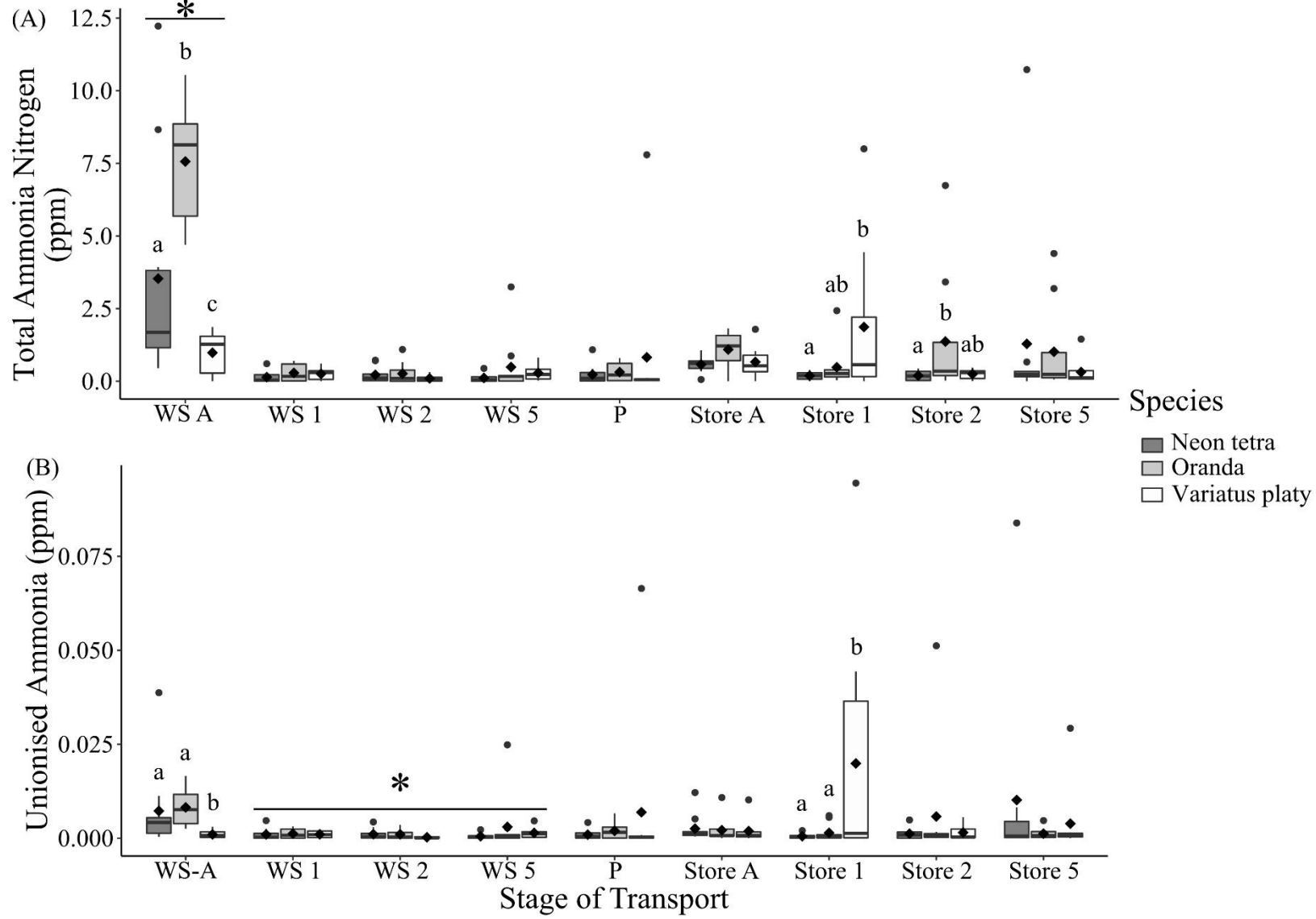
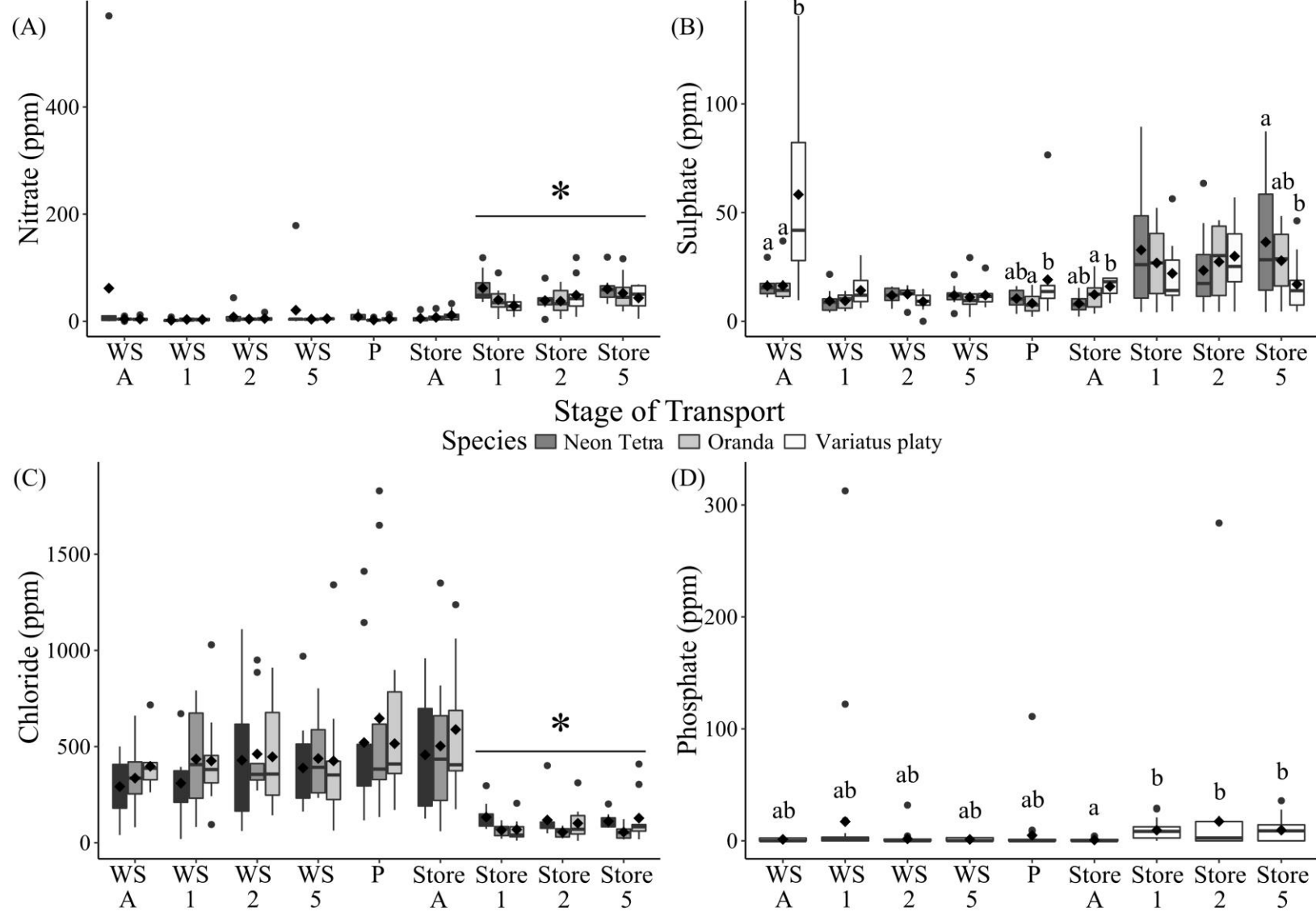


Figure 3



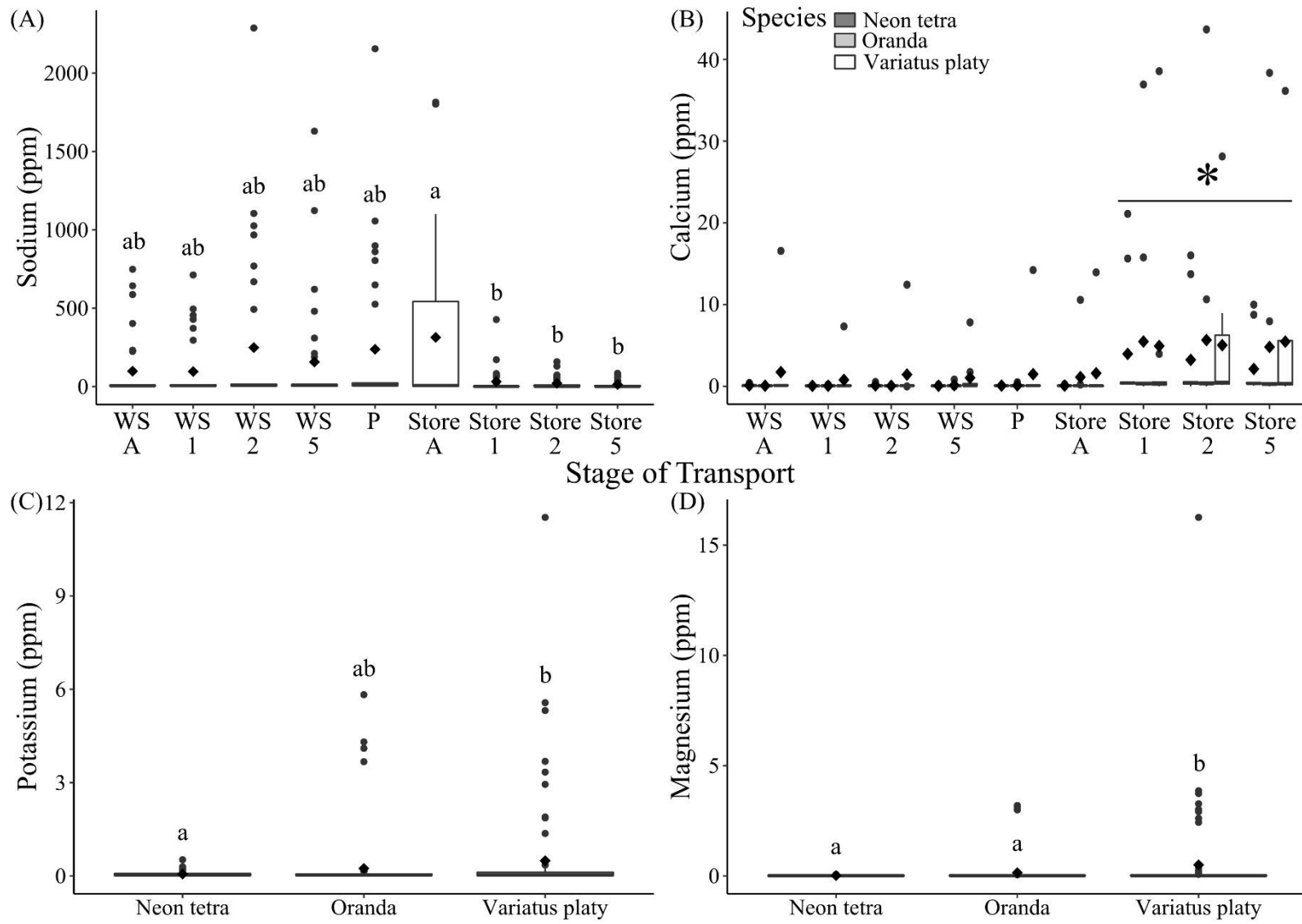
928

Figure 4



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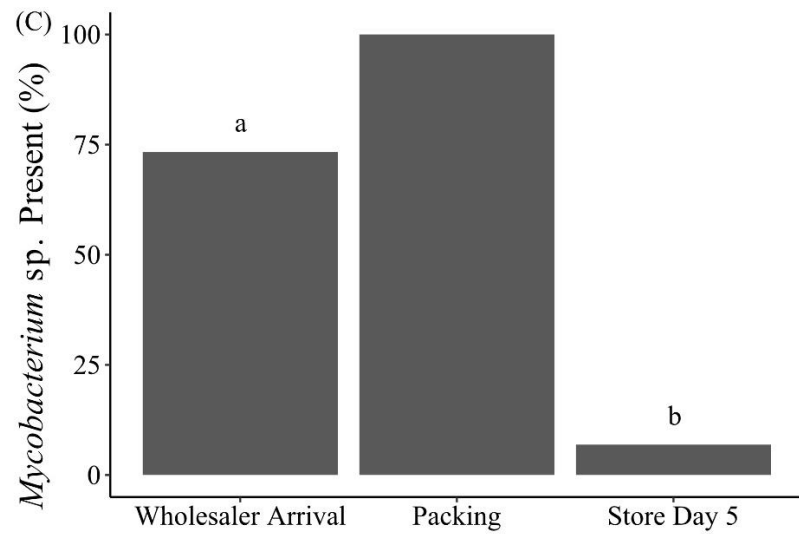
930 Figure 5



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Figure 6

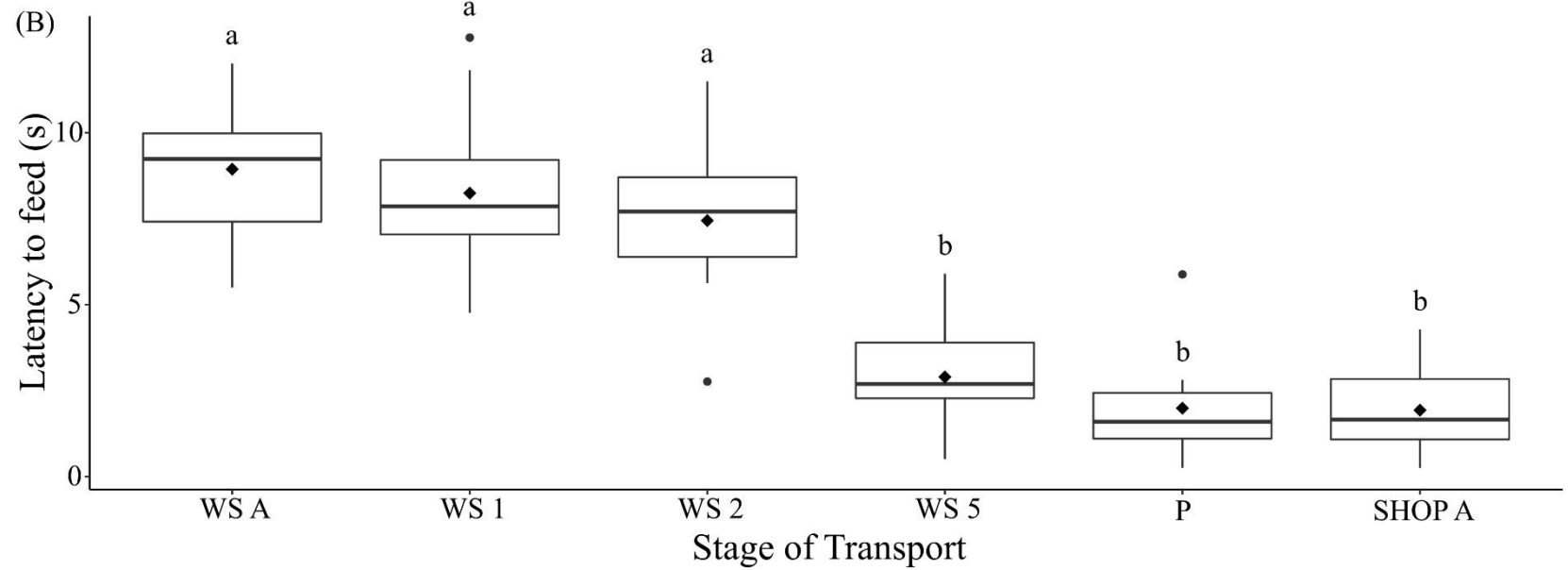
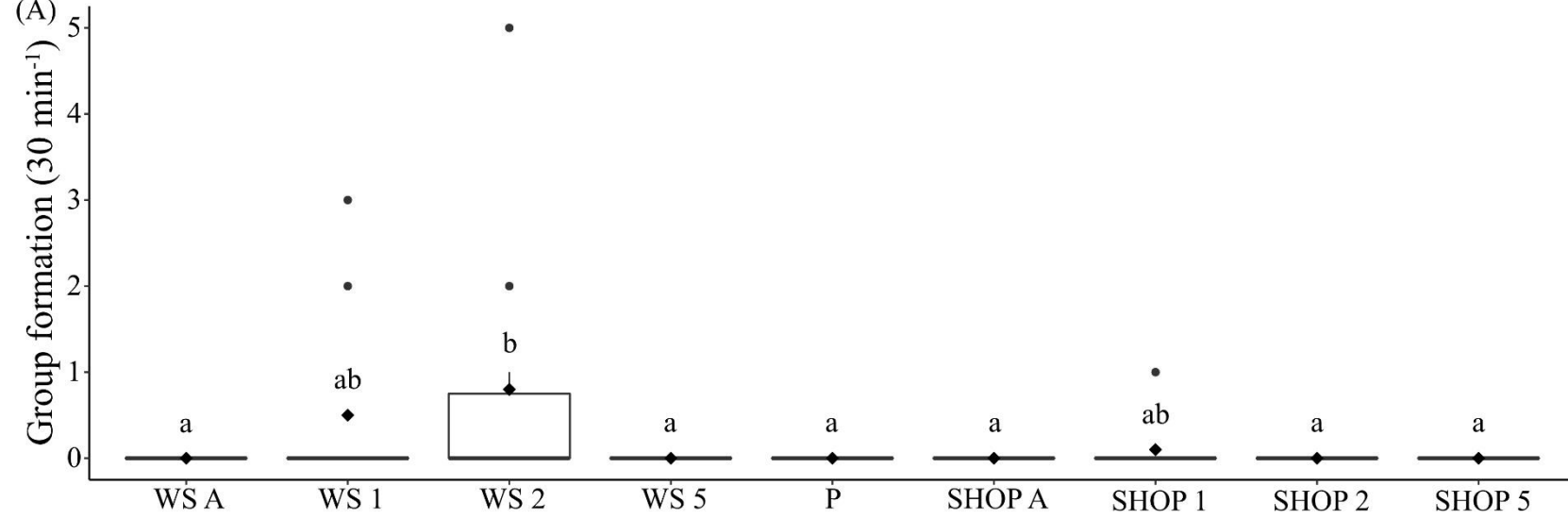


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Figure 7



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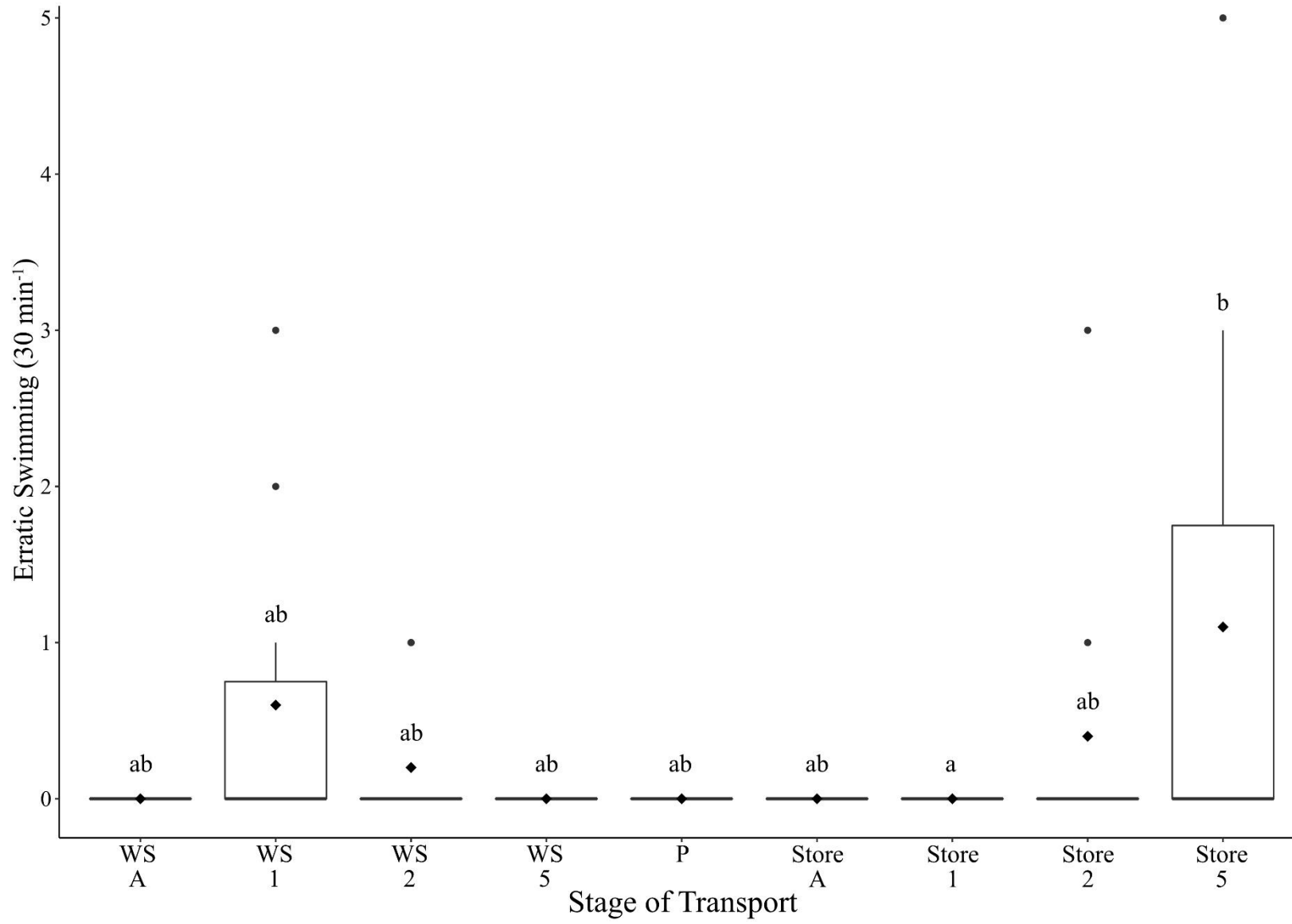
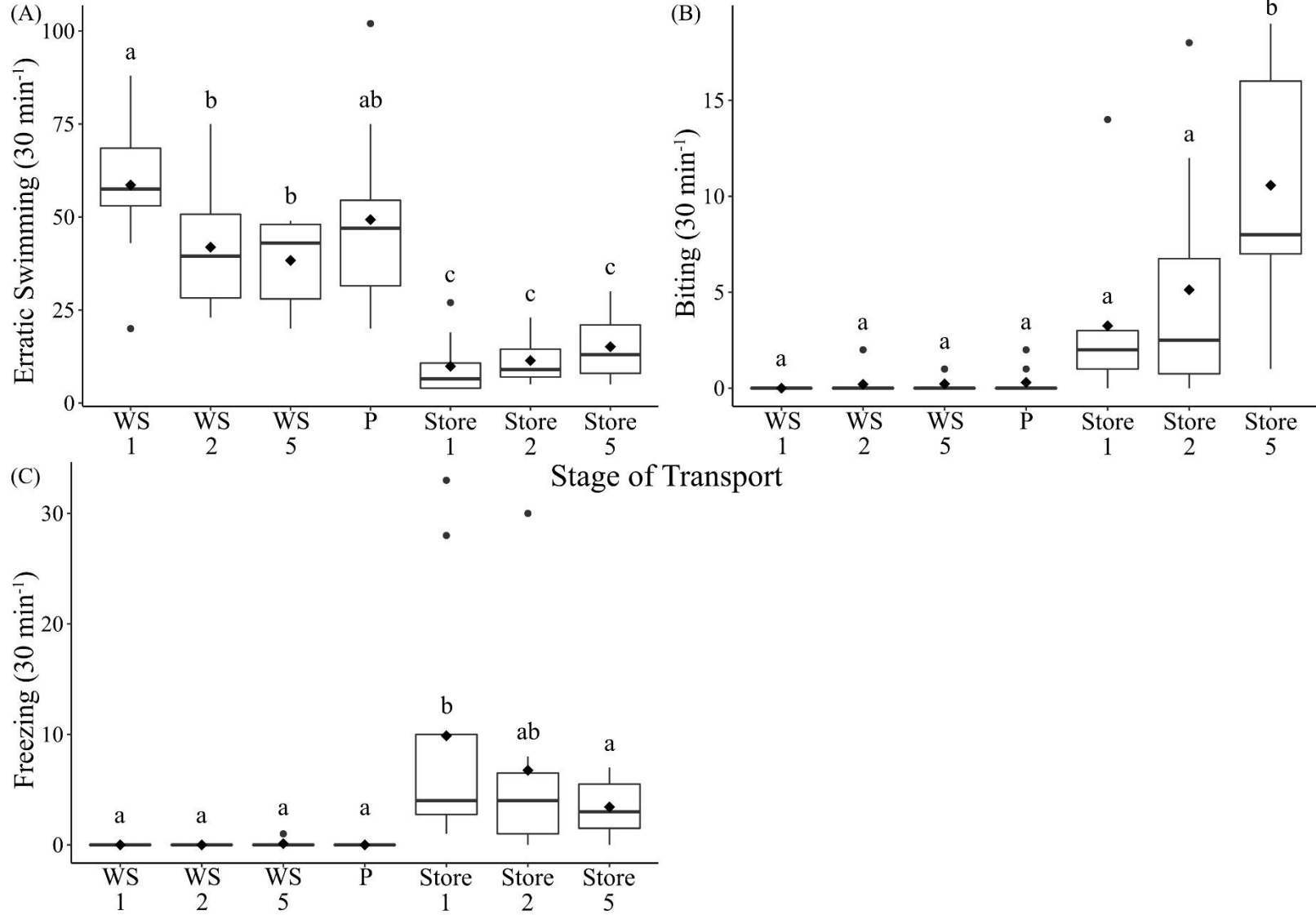
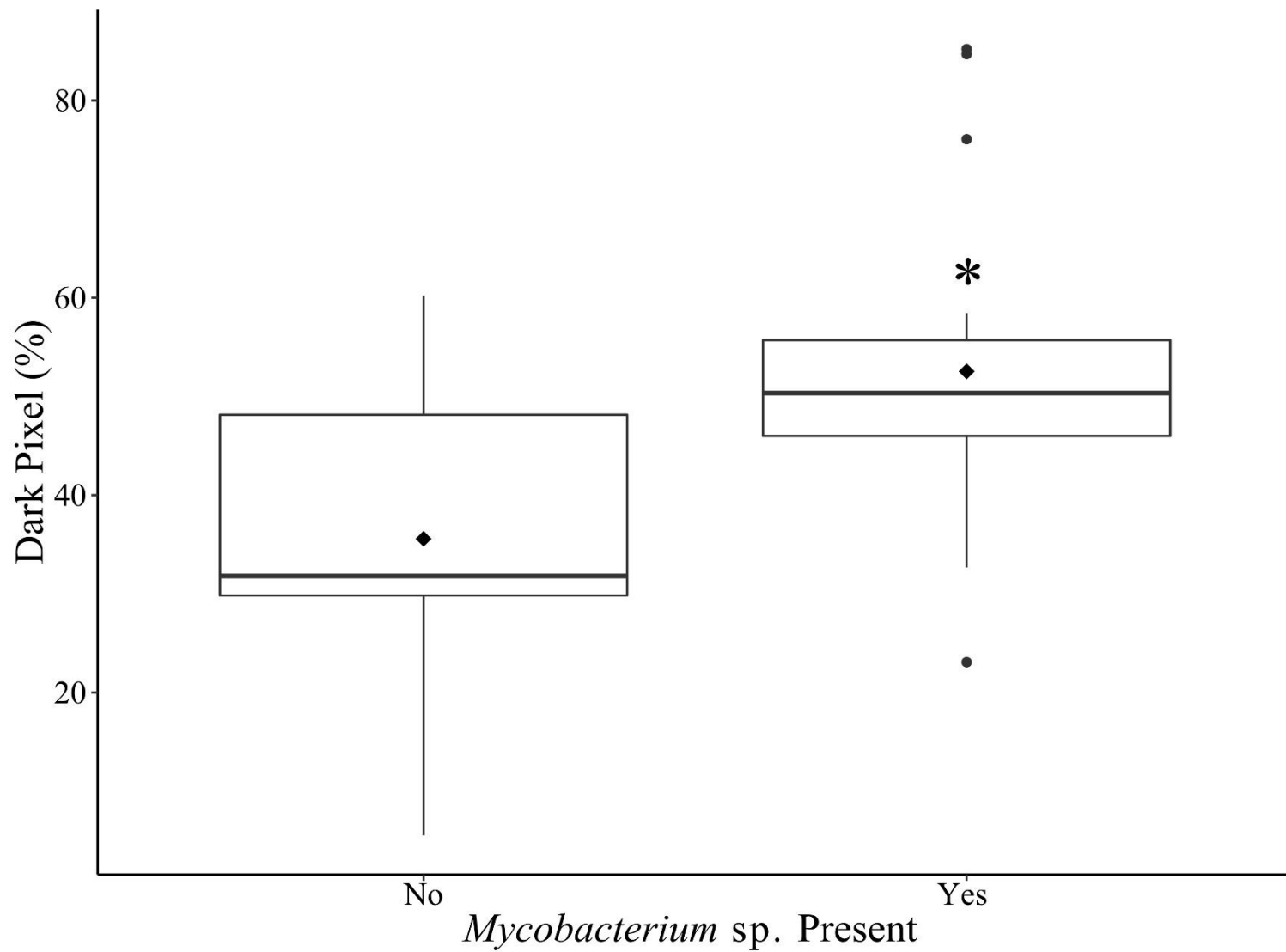


Figure 9





942 **Supplementary Material**

943 Table 1: ANOVA result of best fit model for non-significant behavioural and physiological responses with F values, degrees  
 944 of freedom (df), and p values.

		Transport Stage
Neon tetra		
	Mortality	$\chi^2=54.667$ , $df=48$ , $p= 0.236$
	Ventilation rate	Not visible
	Body condition	No occurrence
	Eye colour	Not visible
	Water cortisol concentrations	$F_{1,4}=0.251$ , $p= 0.642$
	Crowding	$F_{6,44}=0.028$ , $p=0.999$
	Gasping	No occurrence
	Erratic swimming	Not visible
	Freezing	Not visible
	Biting	Not visible
Oranda		
	Mortality	$\chi^2=16.029$ , $df=16$ , $p= 0.451$
	Ventilation rate	$F_{7,41}=1.003$ , $p= 0.612$
	Body condition	$F_{8,62}=1$ , $p= 0.612$
	Eye colour	$F_{3,19}=1.806$ , $p= 0.495$
	Water cortisol concentrations	$F_{1,4}=1.198$ , $p= 0.612$

	Latency to feed	$F_{1,5}=0.511, p=0.765$
	Crowding	$F_{1,4}=1.88, p=0.242$
	Group formation	No occurrence
	Gasping	$F_{1,2}=14.69, p=0.093$
	Freezing	No occurrence
	Biting	No occurrence
Variatus platy		
	Mortality	$X^2=63.358, df=64, p= 0.499$
	Ventilation rate	$F_{7,41}=1.003, p= 0.612$
	Body condition	$F_{8,62}=1, p= 0.816$
	Eye colour	$F_{2,12}=0.259, p= 0.776$
	Water cortisol concentrations	$F_{1,4}=1.06, p= 0.816$
	Latency to feed	$F_{1,5}=2.245, p=0.077$
	Crowding	$F_{1,1}=0.025, p=0.899$
	Group formation	No occurrence
	Gasping	No occurrence