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Monitoring water quality changes and ornamental fish behaviour during commercial transport

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Live transport of fishes is recognised as a major source of stress leading to poor welfare and mortality within the ornamental fish industry. While previous studies have aimed to simulate the stressors experienced by fishes during transport in the laboratory, there is little documented evidence of the actual conditions experienced by fishes under commercial conditions. The aim of this study was to monitor water quality and fish health (physiology and behaviour) through a commercial supply chain for three popular freshwater ornamentals (neon tetra, oranda, variatus platy). Water samples were collected at nine stages of the supply chain beginning at arrival of the fishes at a UK wholesaler from Singapore through their recovery at the wholesaler, subsequent shipment to and recovery at retail stores. Water chemistry was recorded at various points in the transport chain and the water tested for common fish pathogens. Fish health parameters measured included mortality, injuries, waterborne cortisol and behavioural changes. Most water parameters were found to change significantly through the transport chain, including pH, carbonate hardness (KH), chloride (Cl⁻), nitrate (NO₃⁻), sodium (Na⁺), magnesium (Mg²⁺), potassium (K⁺) and calcium (Ca²⁺). Pathogens were detected in the water at each stage of transport, but no disease outbreaks were observed. Mortality was low and was not affected by transport stage. Neon tetras and orandas showed some behavioural changes during transport but the behaviour of variatus platys was more affected by transport stage. The findings of this study identify the changes in water quality experienced by fishes during commercial transport that are often over-looked in simulated studies and confirm the need for species-specific indicators of welfare during commercial transport.

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

• First published study monitoring water quality and fish health (physiology and behaviour) through a commercial ornamental fish supply chain.

• Water quality was high overall but significant changes in water chemistry occurred between different stages of the supply chain.

• Potentially pathogenic microbes were detected in all samples but frequency of detection changed throughout the supply chain.

• Of the measured health indicators, behaviour was found to change the most between different stages of transport, and was also species-specific.
1. Introduction

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

Over the duration of the transport, the quality of the containment water can degrade, resulting in sub-optimal conditions for the fish and high mortality if chemical parameters are not controlled (Harmon, 2009; Lim et al., 2003). Studies simulating commercial transport have shown that the most important water parameters to manage are dissolved oxygen (DO), temperature, pH and ammonia (Bower and Turner, 1982; Harmon, 2009; Ramírez-duarte et al., 2013; Sampaio and Freire, 2016; Silva et al., 2015; Teo et al., 1989). In simulated transport studies, fish are contained in water originating from the same source before, during and after the transport and any fluctuation in water quality is caused by the fish in the transport bag. However, within commercial settings, ornamental fishes are exposed to water from many different sources from their time at either aquaculture farms or in the wild, to their final arrival in a home aquarium. The water quality at different stages of the transport chain is likely to change abruptly, not only due to excretion from the fish but also from different water sources used by the various stakeholders and any measures used to control water quality and health of the fish. Elements such as calcium (Ca²⁺), potassium (K⁺), magnesium (Mg²⁺),
and sodium (Na⁺) are important for osmoregulation (Baldisserotto et al., 2007; Evans, 2008) whereas aluminium (Al), copper (Cu) and lead (Pb) are toxic in excess to fishes (Shuhaimi-Othman et al., 2015; Spry and Wiener, 1991). Only a few studies have assessed changes in water quality and fish health for a complete transport chain (i.e. from the farm or time of capture to the retail store) and the range of water quality parameters measured in these studies was limited (Baldisserotto et al., 2014; Correia et al., 2011; Rodrigues et al., 2013).

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

In addition to the lack of information on change in water quality during commercial transport, little is known about the cumulative effect of repeated transport and exposure to water of varying quality on the stress and welfare of ornamental fishes. Most studies assessing stress experienced in this context measure a range of stress indicators such as plasma cortisol, lactate and glucose levels (Ferreira et al., 2017; Inoue et al., 2005; Salbego et al., 2015) and the techniques involved are invasive
(Abreu et al., 2014; Azambuja et al., 2011; Zanuzzo et al., 2017). However, in small ornamentals, taking a blood sample in order to measure these indicators of stress often requires sacrificing the fish. Less invasive indicators that could potentially be used include levels of cortisol in the water (Ellis et al., 2004; Ruane and Komen, 2003), changes in behaviour such as increased aggression (Braithwaite and Ebbesson, 2014; Huntingford et al., 2006; Weber, 2011), increased ventilation rate as an indicator of increased metabolic rate (Brydges et al., 2009; Huntingford et al., 2006; Portz et al., 2006) and darkening of the fish skin and eye (Backström et al., 2015; Freitas et al., 2014; Huntingford et al., 2006). However, we could find no study that assessed transport stress in ornamental fishes using non-invasive sampling techniques on a commercial scale.

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

2. Materials and Methods

2.1. Study Animals

The commercial transport of three ornamental fish species was studied to assess changes in water chemistry, waterborne pathogens and fish physiology and behaviour. The three species investigated were neon tetra (*Paracheirodon innesi*), small oranda goldfish (*Carassius auratus*) and yellow strain variatus platy (*Xiphophorus variatus*). Ten bags of each species were randomly selected and sampled over a period of 10 weeks starting from the fishes’ arrival at a UK wholesaler. The fishes were all reared in farms in Singapore and prior to transport to the UK food was withheld for 24 h prior before fish were packed in polyethylene bags (neon tetra: 20 x 16.5 x 70 cm; oranda and variatus platy: 35.5 x 70 cm) containing water (neon tetra: 2.7 l; oranda: 1.4 l and variatus platy: 4 l) and 25 ml of liquid
zeolite with the air space at the top of the bag filled with pure oxygen. The stocking density was 148 fish l\(^{-1}\) for neon tetra, 72 fish l\(^{-1}\) for oranda and 45 fish l\(^{-1}\) for variatus platy. The bags were then air-tight sealed using a metal wire and placed into a polystyrene box insulated with newspapers along with a variable number of other bags, then into a cardboard box. The bags of fish were air-shipped to a UK airport where they went through customs and were then transported by road to an aquatics wholesaler. The total transport duration was approximately 32 h. On arrival at the wholesaler, water samples were taken from the bags for later analysis of water chemistry, waterborne pathogens and waterborne cortisol concentrations (see details below). Samples for water cortisol were frozen immediately on dry ice. Bags containing the remaining water and fish were then placed individually into aerated tanks (76 cm x 38 cm x 38 cm) containing 100 l of water at 24º C. At this point the antibiotic tetracycline was added to all the tanks (20 mg l\(^{-1}\)) so that it was fully dissolved by the time the fish were added. The bags were floated in the tank for approximately 30 min to allow temperatures to equilibrate, and then the bags were opened and briefly submerged to introduce tank water into the bag. Approximately 30 min later the fish were released into the tank. During recovery at the wholesaler, fish were kept under a 10:14 light/dark regime with a light fitted with a UV filter. Fish were fed three times daily *ad libitum* with Vitalis tropical or goldfish flake (World Feeds Ltd, UK) by members of wholesaler staff, who at this point would also remove any dead fish. On day 2 of recovery at the wholesaler, methylene blue (0.1%) was added to all the tanks (1 ml l\(^{-1}\)). The fish then spent 7 days at the wholesaler, where water samples were taken on days 1, 2 and 5 for later analysis of water chemistry, prior to being transported to retail stores.

For transport to regional retail stores, the fish underwent the same packing process as for international transport, but no zeolite was added to the bag and food was not withheld prior to bagging. The loading density was 22 fish l\(^{-1}\) for neon tetra, 7 fish l\(^{-1}\) for oranda and 11 fish l\(^{-1}\) for variatus platy. Water samples were taken from each bag before it was sealed for later analysis of water chemistry, waterborne pathogens and water cortisol concentrations. Ten bags of each species were transported by road to five different retail stores (each store received two bags per species over a period of 10 weeks), with a journey time of less than 6 h. On arrival at the retail store, water samples
were again taken from each bag for water chemistry and water cortisol. Each bag was then placed into a separate recovery tank, and the fishes were acclimated using the same process as used at the wholesaler. Fish were kept under a 12 h light/dark regime. They were fed twice daily *ad libitum* (neon tetra: Love Fish tropical fish flakes, oranda: Love Fish coldwater pellets by Pets at Home Group Plc, variatus platy: Love Fish temperate fish flakes) by retail store staff, who at this point also removed any dead fish. Water samples were taken on days 1, 2 and 5 of recovery at the stores for analysis of water chemistry and on day 5 only for analysis of waterborne pathogens. In addition to the collection of water samples, video recordings (30 min) were made using cameras (WiMiUS 4K 16MP Action) mounted on monopods and set at 720 P (progressive scan) and 120 fps (frames per second). This was carried out on a number of occasions: 1) upon arrival at the aquatics wholesaler, 2) on days 1, 2 and 5 of recovery at the wholesaler, 3) before packing for transport to the retail stores, 4) on arrival at the retail stores and 5) on days 1, 2 and 5 of recovery at the stores.

### 2.2. Water Chemistry

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

A Dionex ICS-1100 ion chromatography system was used to determine chloride (Cl⁻), phosphate (PO₄³⁻), sulphate (SO₄²⁻) and nitrate (NO₃⁻) concentration. Conditions were: column: Dionex IonPac AS14a (4mm*250mm); delivery speed: 4 ml min⁻¹; delay volume: 125 μl; flow rate: 1 ml min⁻¹; eluents: 0.25 mM sodium carbonate (Na₂CO₃) and 0.25 mM sodium bicarbonate (NaHCO₃) in a ratio of 8:1. Total run time was 20 min. For metal concentrations, a Perkin Elmer Avio-500 ICP-
OES was used to determine Al, Ca$^{2+}$, Cu, K$^+$, Mg$^{2+}$, Na$^+$ and Pb. A multi-element standard (Fisher Scientific, product code 1009-2633) was used for calibration. Filtered samples were acidified with nitric acid (1%) prior to analysis. The wavelengths used were: Al (396.153 nm), Ca$^{2+}$ (317 nm), Cu (324.752 nm), K$^+$ (766 nm), Mg$^{2+}$ (285 nm), Na$^+$ (589.592 nm), Pb (220.353 nm).

Total ammonia nitrogen levels were quantified using a microplate colourimetric procedure (Bower and Holm-Hansen, 1980). Standards were made by a dilution of 1 mM ammonium chloride (NH$_4$Cl). Standards and samples were run in triplicate. The un-ionised ammonia (NH$_3$) content was calculated from the total ammonia nitrogen (TAN) values, the pH and temperature (°C) (California Water Boards Agency, 2011).

2.3. Waterborne Pathogens

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence (5’-3’)</th>
<th>Reverse Sequence (5’-3’)</th>
<th>Annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas spp.</td>
<td>5’-TCCGTTGGATATGGCTCTTC-3’</td>
<td>5’-GCCTACCGATGTCTGAA-3’</td>
<td>58° C</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>5’-GACGGGTGAGTAATGCCTA-3’</td>
<td>5’-CACTGTTGTTCTCTTCTATA-3’</td>
<td>63° C</td>
</tr>
<tr>
<td><em>Flavobacterium columnare</em></td>
<td>5’-GCAGGATGACGGTCCTATGG-3’</td>
<td>5’-TTAACCTGACACCTACAGGC-3’</td>
<td>58° C</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp.</td>
<td>5’-GTACAGTTGCTTCAGACGTATC-3’</td>
<td>5’-ACGTTGATTTTCATCACGTTG-3’</td>
<td>52° C</td>
</tr>
<tr>
<td>Mycobacterium spp.</td>
<td>5’-CTTTCTAAGGAGACCACGA-3’</td>
<td>5’-TTGCTTAGATGCTCGCAACC-3’</td>
<td>58° C</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>5’-CGGACGGGTGAGTAATGTCT-3’</td>
<td>5’-TTAGCGGTTGCTCTTCTTG-3’</td>
<td>65° C</td>
</tr>
<tr>
<td>Acanthamoeba spp.</td>
<td>5’-GGCCCAGATCGTTTACCGTGAA-3’</td>
<td>5’-TCTCAACAAGCTGCTAGGGAGTCA-3’</td>
<td>62° C</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em></td>
<td>5’-AAGAAGCACCAGCTACCC-3’</td>
<td>5’-CCATGACACCGTCTTAC-3’</td>
<td>53° C</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>5’-GCGATTGATGGATACGGTT-3’</td>
<td>5’-CAAGCCTTGACGAACTAAAGC-3’</td>
<td>55° C</td>
</tr>
</tbody>
</table>
2.4. Fish physiology and behaviour

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

2.4.1. Waterborne Cortisol

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

2.4.2. Behaviour

Behavioural Observation Research Interactive Software (BORIS) (Friard and Gamba, 2016) was used to analyse behaviour displayed by the fish (Table 2). To determine reliability of analyses, 10% of the video were analysed blind by a second observer and concordance was found to be high.
between observers (Kendall’s W coefficient: 0.96, p<0.01). Randomly selected individual fish were tracked for 3 min, and this was repeated consecutively from the start to the end of the video such that 10 individuals were tracked. Displayed behaviours for each focal fish were totalled to obtain either a bag or tank score depending on the stage of transport. For neon tetras, only gasping, crowding and group formation were visible on the videos due to the small size of the fish. On arrival at the wholesaler while the fish were still in the bag, only gasping, crowding and group formation were measured due to visibility at high stocking densities. Only behaviours that were visible at specific stages of transport were included in statistical analyses.
Table 2. Ethogram of the behaviours recorded.

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Description</th>
<th>Relation to welfare</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaping</td>
<td>Approximate per cent of fish gasping at the surface of the water.</td>
<td>Gasping behaviour indicates low-quality water, specifically, low levels of DO (Kramer, 1987).</td>
</tr>
<tr>
<td>Crowding</td>
<td>Approximate per cent of fish grouped close (within 1 body length) together and swimming in the same direction.</td>
<td>Crowding increases with stress and can be a result of a perception of threat (Schreck et al., 1997).</td>
</tr>
<tr>
<td>Group formation</td>
<td>All fishes in the tank forming multiple schools with 30 cm between the groups.</td>
<td>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).</td>
</tr>
<tr>
<td>Biting</td>
<td>Occurrence of fish biting or nipping a conspecific.</td>
<td>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).</td>
</tr>
<tr>
<td>Erratic swimming</td>
<td>Occurrence of rapid swimming and direction change in the absence of being chased.</td>
<td>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).</td>
</tr>
<tr>
<td>Latency to feed</td>
<td>Duration (s) from the time food is dropped in the tanks to the time 90% of the fish are feeding.</td>
<td>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).</td>
</tr>
<tr>
<td>Freezing</td>
<td>Duration (s) fish spend immobile.</td>
<td>Freezing is considered a fear and stress-related behaviour (Braithwaite and Salvanes, 2005; Koolhaas et al., 1999).</td>
</tr>
<tr>
<td>Ventilation rate</td>
<td>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).</td>
<td>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).</td>
</tr>
</tbody>
</table>
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; Neitze 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.
2.6. Statistical Analyses

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

The effect of pathogen presence on behaviour and physiological responses was determined by model analysis. For each response variable a general or generalised linear model was carried out as
described above with presence of each pathogen set as explanatory variables. Model selection via AIC
was carried out as described above to identify pathogens that significantly affected the response
variable. T-tests were carried out for each selected pathogen to determine the effect of pathogen
presence on response behaviour.

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

3. Results

3.1. Water Chemistry

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Transport Stage</th>
<th>Species</th>
<th>Transport Stage*Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO</td>
<td>$F_{8,218}=132.43$, $p&lt;0.001$</td>
<td>$F_{8,218}=12.69$, $p&lt;0.001$</td>
<td>$F_{16,218}=4.88$, $p&lt;0.001$</td>
</tr>
<tr>
<td>Temperature</td>
<td>$F_{8,220}=11.5$, $p&lt;0.001$</td>
<td>$F_{2,220}=42.3$, $p&lt;0.001$</td>
<td>$F_{16,220}=9.3$, $p&lt;0.001$</td>
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<td>pH</td>
<td>$F_{8,220}=11.175$, $p&lt;0.001$</td>
<td>$F_{2,220}=7.78$, $p=0.001$</td>
<td>$F_{16,220}=1.43$, $p=0.214$</td>
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Table 1: 315

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<th>Ion</th>
<th>F(6,236)=3.58, p=0.002</th>
<th>F(2,236)=0.503, p=0.781</th>
<th>F(61,220)=0.378, p=0.998</th>
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<tr>
<td>KH</td>
<td>F(6,236)=3.58, p=0.002</td>
<td>F(2,236)=0.503, p=0.781</td>
<td>F(61,220)=0.378, p=0.998</td>
</tr>
<tr>
<td>TAN</td>
<td>F(6,236)=23.64, p&lt;0.001</td>
<td>F(2,236)=9.65, p&lt;0.001</td>
<td>F(61,220)=4.55, p&lt;0.001</td>
</tr>
<tr>
<td>NH₃</td>
<td>F(6,236)=3.41, p=0.059</td>
<td>F(2,236)=0.245, p=0.874</td>
<td>F(61,220)=2.32, p=0.008</td>
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<tr>
<td>NO₃⁻</td>
<td>F(6,236)=25.22, p&lt;0.001</td>
<td>F(2,236)=3.41, p=0.059</td>
<td>F(61,220)=1.10, p=0.543</td>
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<tr>
<td>SO₄²⁻</td>
<td>F(6,236)=4.73, p&lt;0.01</td>
<td>F(2,236)=1.44, p=0.237</td>
<td>F(61,220)=0.70, p=0.879</td>
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<td>Cl⁻</td>
<td>F(6,236)=20.49, p&lt;0.01</td>
<td>F(2,236)=0.84, p=0.571</td>
<td>F(61,220)=2.69, p=0.018</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>F(6,236)=4.73, p&lt;0.01</td>
<td>F(2,236)=1.44, p=0.237</td>
<td>F(61,220)=0.70, p=0.879</td>
</tr>
<tr>
<td>Na⁺</td>
<td>F(6,236)=7.21, p&lt;0.01</td>
<td>F(2,236)=3.34, p=0.071</td>
<td>F(61,220)=0.47, p=0.998</td>
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<td>Ca²⁺</td>
<td>F(6,236)=4.42, p&lt;0.001</td>
<td>F(2,236)=4.22, p=0.035</td>
<td>F(61,220)=0.26, p=0.998</td>
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<td>K⁺</td>
<td>F(6,236)=0.60, p=0.83</td>
<td>F(2,236)=2.59, p=0.012</td>
<td>F(61,220)=0.53, p=0.998</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>F(6,236)=0.63, p=0.875</td>
<td>F(2,236)=9.46, p&lt;0.01</td>
<td>F(61,220)=0.81, p=0.826</td>
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</table>

DO was higher on arrival at both the wholesaler and the retail store and was higher in the neon tetra water on arrival at the wholesaler than in the oranda and variatus platy water. DO remained higher in the neon tetra tanks than in the oranda tanks on day 1 and 2 of recovery at the wholesaler (Fig. 1A). Water temperature was affected by transport stage (Table 3) and species. The water temperature experienced by neon tetras did not change throughout their transport, whereas both orandas and variatus platy experienced a decrease in temperature at the retail stores (Fig. 1B). For all species, water pH was significantly lower on arrival at the wholesaler than at subsequent transport stages and throughout the pH in the neon tetra water was higher than that of oranda water (Fig. 2A). KH was significantly higher on arrival at the wholesaler than in the water fish were packed in for transport to the retail stores (Fig. 2B; Table 3). Species and the interaction between species and transport stage was not significant (Table 3).
TAN was significantly affected by stage of transport and species and the two factors interacted significantly (Fig. 3A; Table 3). TAN water levels on arrival at the wholesaler were significantly higher in neon tetra and oranda bags than during the subsequent transport stages (Fig. 3A). On day 1 of recovery at the store, TAN was higher in variatus platy tanks than in neon tetra tanks; on day 2 of recovery at the store, TAN was higher in oranda tanks than in neon tetra tanks (Fig. 3A). NH$_3$ was lower during recovery at the wholesaler than during the other transport stages with inter-specific differences in NH$_3$ dependent upon transport stage (Fig. 3B; Table 3).

There was a significant effect of transport stage on NO$_3^-$ and the effect of species approached significance (Fig. 4A; Table 3). For all three species, NO$_3^-$ concentrations were significantly higher in the tank water during recovery at the stores than during the previous transport stages. SO$_4^{2-}$ concentrations were affected by the stage of transport and species (Fig. 4B; Table 3). SO$_4^{2-}$ concentrations in the water of neon tetra bags were significantly lower on arrival at the retail stores than in the tanks during recovery at the stores. Variatus platy were exposed to significantly higher SO$_4^{2-}$ concentrations in bags on arrival at the wholesaler than in the tanks during recovery at the wholesaler and packing for shipment to the stores (Fig. 4B). For all three species, Cl$^-$ concentrations were lower in tanks during recovery at the retail store than at the wholesaler (Fig. 4C; Table 3). PO$_4^{3-}$ concentrations were significantly lower in bag water on arrival at the stores than in tanks during recovery at the stores (Fig. 4D; Table 3). For all three species, water Na$^+$ concentration was significantly higher on arrival at the retail store than during recovery at the stores (Fig. 5A; Table 3) and water Ca$^{2+}$ concentrations were significantly lower on arrival at the retail store than during recovery at the store (Fig. 5B). Concentrations of K$^+$ were lower in the water of neon tetra than in the water of variatus platy but were not different to the water for oranda (Fig. 5C; Table 3) and Mg$^{2+}$ levels were higher in variatus platy water than in the neon tetra water or the oranda water (Fig. 5D; Table 3). Al, Cu and Pb were below detection limits (<0.008 ppm).

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

### 3.3. Fish physiology and behaviour

In neon tetras only two behaviours showed significant changes through transport stage. Group formation behaviour was greater on day two of recovery at the wholesaler than at the other stages of transport, except for day 1 of recovery at the wholesaler and at the store ($F_{8,60}=4.711$, $p=0.004$; Fig. 7A). Latency to feed was significantly lower during recovery at the retail stores than during recovery at the wholesaler ($F_{1,33}=19.404$, $p<0.001$; Fig. 7B). In oranda, the occurrence of erratic swimming was highest on day 5 of recovery at the store ($F_{1,8}=5.66$, $p=0.003$; Fig. 8). In variatus platy, occurrence of erratic swimming significantly decreased between day 1 of recovery at the wholesaler and day 2 and 5 of recovery at the wholesaler (Fig. 9A). Variatus platy displayed significantly less erratic swimming during recovery at the store than at the wholesaler ($F_{6,38}=10.269$, $p<0.001$; Fig. 9A). Biting behaviour was significantly affected by the transport stage with a general increase seen in the occurrence of biting behaviour through transport stages and the occurrence of biting was significantly higher at day 5 of recovery at the store than the previous stages ($F_{6,38}=7.618$, $p<0.001$; Fig. 9B).
occurrence of freezing behaviour was significantly higher on day 1 of recovery at the store than all the
other recorded stages of transport, except for day 2 of recovery at the store (F\textsubscript{6,38}=4.569, p=0.002; Fig.
9C). Erratic swimming, biting and freezing behaviour could not be observed on arrival at the
wholesaler or the store, therefore these time points were excluded from the analysis. No other
significant changes were recorded in behavioural and physiological measures (Supplementary
Material, Table 1). In orandas, the presence of \textit{Mycobacteria} spp. in the water resulted in a
significantly darker eye colour (t-test=2.611, p=0.026; Fig. 10). No other pathogen was found to have
a significant effect on any of the recorded behaviour and health measures (p<0.05).

Distance-based linear models revealed that, in neon tetra, 56.03\% of variation in the latency to
feed was associated with \textit{NO}_3^-, \textit{Cl}^-, \textit{Na}^+, \textit{PO}_4^{3-} and \textit{Mg}^{2+} concentrations (Table 4) changes in group
formation were not attributable to water chemistry. Copper and TAN explained 32.19\% of the
variation in erratic swimming behaviour in oranda whereas in variatus platy, 62.52\% of variation in
erratic swimming was explained by DO, \textit{Cl}^-, \textit{NO}_3^- and \textit{K}^+ (Table 3). Variation in biting behaviour
(35.26\%) displayed by variatus platy was explained by temperature, pH and \textit{NH}_3 and variation in
freezing behaviour (18.15\%) was explained by \textit{NO}_3^- and pH (Table 4).
Table 4. Results for behavioural variation in neon tetra, oranda and variatus platy showing the chemical parameters selected via DistLM indicating Pseudo-F, p value and residual degrees of freedom (df).

<table>
<thead>
<tr>
<th>Species</th>
<th>Behaviour</th>
<th>DistLM</th>
<th>Contribution (%)</th>
<th>Pseudo-F</th>
<th>p value</th>
<th>Residual df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neon tetra</td>
<td>Latency to feed</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₃⁻</td>
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<td>56</td>
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<tr>
<td>Cl⁻</td>
<td>14.899</td>
<td>14.510</td>
<td>0.001</td>
<td>55</td>
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<td></td>
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<tr>
<td>Na⁺</td>
<td>5.515</td>
<td>5.843</td>
<td>0.013</td>
<td>54</td>
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<td>PO₄³⁻</td>
<td>3.585</td>
<td>4.010</td>
<td>0.044</td>
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<tr>
<td>Mg²⁺</td>
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<td>4.037</td>
<td>0.056</td>
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<td>Oranda</td>
<td>Erratic swimming</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Cu</td>
<td>29.323</td>
<td>33.191</td>
<td>0.005</td>
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<td>TAN</td>
<td>2.872</td>
<td>3.346</td>
<td>0.059</td>
<td>79</td>
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<tr>
<td>Variatus platy</td>
<td>Erratic swimming</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DO</td>
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<td>Cl⁻</td>
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<td>22.285</td>
<td>0.001</td>
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<tr>
<td>NO₃⁻</td>
<td>2.508</td>
<td>3.596</td>
<td>0.062</td>
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<tr>
<td>K⁺</td>
<td>2.283</td>
<td>3.412</td>
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<td>12.462</td>
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<td>pH</td>
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<td>NH₃</td>
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<tr>
<td>Cl⁻</td>
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<td>2.026</td>
<td>0.133</td>
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<td>Freezing</td>
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<td>6.444</td>
<td>0.026</td>
<td>60</td>
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<td>pH</td>
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<td>6.094</td>
<td>0.022</td>
<td>59</td>
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</table>
4. Discussion

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

Water quality was high from arrival at the wholesaler to recovery at the stores. Throughout the stages of transport, oxygen was on average above 100% saturation. The mean water pH was 6.7 with a few instances where pH dropped below 5.5. The range in water pH between stores was large due to the geographical distribution of the stores. Small daily fluctuations in pH are unlikely to have a long term effect on fish, although this will be species-dependent (Cecil, 1999; Oliveira et al., 2008; Roberts and Palmeiro, 2008). High levels of NH$_3$ are toxic to fishes and can lead to mortality (LC50 toxicity $\sim$2.79 mg l$^{-1}$ in freshwater fishes at pH 8 and 20 °C based on 32 freshwater species but LC50 is different between species) (Randall and Tsui, 2002, USEPA, 1984). Average levels of NH$_3$ were 0.003 mg l$^{-1}$ and any peaks in NH$_3$ were still well below toxic levels (recorded peaks <0.1 mg l$^{-1}$).

Liquid zeolite (25 ml) was used during the international leg of the transport chain to regulate ammonia concentrations. The ammonia concentrations in the present study (0.005 mg l$^{-1}$) are in line with the zeolite treatment in a study by Bower and Turner (1982) (0.007 mg l$^{-1}$) who compared NH$_3$ levels in bags containing 0-20 g zeolites following a 24 h simulated transport with 10 or 20 goldfish (C. auratus) in 500 ml water. Elevated NO$_3^-$ levels (> 50 mg l$^{-1}$) can lead to poor growth, lethargy,
anorexia and opportunistic infections (Roberts and Palmeiro, 2008). The levels recorded in this study were on average <17 ml l⁻¹, well below this threshold.

The present study measured a range of chemical elements including NO₃⁻, Cl⁻, Ca²⁺ and trace metals. The results showed that the levels of some chemical elements in the water fluctuated between species (potassium and magnesium), between stages of transport (PO₄³⁻ and sodium) or between both species and stages (NO₃⁻, SO₄²⁻, Cl⁻ and Ca²⁺). Similar data were collected in a previous study which compared ion levels in water between multiple water sources of a commercial supply chain in Nile tilapia (Oreochromis niloticus) (Colt et al., 2011). This fluctuation in chemical elements highlights that ornamental fishes can be exposed to different water qualities through the supply chain. Fluctuation in water chemistry between times points is likely due to daily or geographical fluctuations but further research is needed. It is unclear why water chemistry was different between species within a time point as they were always exposed to water from the same source. Differences in feed composition or fish physiology might result in the observed fluctuation however this requires further investigation. In this study, the trace metals measured were below the detection limit for the majority of samples; the few samples that did contain detectable levels were within levels considered safe for freshwater fishes (Poléo, 1995; Roberts and Palmeiro, 2008). Overall whilst water quality was appropriate for each species at all stages of transport, fishes experienced significant changes in water chemistry between different stages; the effect of this fluctuation in water quality demands further examination.

The water was tested for the presence of common bacterial pathogens and a free-living amoeba which can harbour pathogens. None of the water samples collected were pathogen-free with E. coli, F. columnare and V. anguillarum pathogens being found in over 90% of the samples. The number of samples which tested positive for Aeromonas spp., Pseudomonas spp. and Mycobacterium spp. was significantly lower on day 5 at the retail stores than on arrival at the wholesaler. However, there was no clear pattern in the presence/absence of the other pathogens across the transport chain indicating that the microbial control measures taken by the wholesaler (tetracycline) and the stores (UV light filter) reduce some of the pathogenic microbial load in the supply chain. Rose et al. (2013) found bacteria isolated from 15 genera in imported ornamental fishes and associated water, however their
detection of common fish pathogens was low for some genera (e.g. *Vibrio* spp.). Other studies, such as Trust and Bartlett (1974) and Zanoni et al. (2008), also detected pathogenic genera (e.g. *Vibrio* spp. and *Mycobacterium* spp.) in ornamental fishes or their water that were either recently imported or sold in retail stores. The difference in detected genera between studies shows how variable the bacterial load can be in supply chains. Many importers receive fishes from several farms and continents; while at the importer, cross-contamination can occur between species from different origins via husbandry practices resulting in the introduction of potential pathogens in non-contaminated water. Further research is needed to better understand the prevalence and transfer of pathogens into the ornamental fish trade.

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

Fishes can become susceptible to many pathogens during periods of high stress as a result of a weakened immune system (Scholz, 1999; Yanong, 2010). It is therefore important to recognise signs
of stress and poor welfare in ornamental fishes during commercial transport which may indicate increased susceptibility to waterborne pathogens. Mortality levels in our study were low for all three species and were not affected by transport stage. Other studies suggest that mortality is likely to be higher during recovery periods than immediately post-transport (Ali et al., 1989; Kilgore et al., 2009) so it may be of benefit in the future to monitor mortality at retail stores over a longer time period. Of course, retail stores are usually not the end-point of the journey of an ornamental fish and understanding mortality effects may need to include the journey from retailer to owner and recovery in home aquaria.

Out of the recorded stress measures, behaviour was found to change the most between stages of transport. Due to the small size of neon tetra, observing individual’s behaviour was not possible, therefore, studying behaviour of this species in a commercial setting is challenging. Oranda had low levels of activity at most stages of the transport chain although a small increase in erratic swimming was seen during recovery at the stores. This increase in erratic swimming was small, however and may not represent a biologically relevant change. In contrast, variatus platy displayed the greatest changes in behaviours across the transport chain. The high levels of erratic swimming at the wholesaler may be associated with the activity of wholesaler staff, which were observed to disturb the fish. Housing variatus platy in tanks in a quieter area of the wholesaler facilities could therefore help reduce stress for this species. At the retail stores, erratic swimming was much lower in variatus platy compared to at the wholesalers which could be due to differences in housing conditions (e.g. stocking density, lighting, enrichment). In contrast, biting increased during recovery at the stores. Biting occurs in particular during the establishment of hierarchies and can result in stress and injury (Noble et al., 2012; Pitcher, 1986). This increase in biting behaviour in variatus platy at the retail stores was accompanied by a decrease in freezing behaviour, likely as a result of the fish avoiding bites and aggression from conspecifics (Braddock, 1945; Scott and Currie, 1980). The decrease in erratic swimming behaviour from the wholesaler to the store may indicate a reduction in stress following transport. It is possible that platys were still experiencing transport-related stress during recovery at the wholesaler. As this stress started to reduce during recovery at the stores, establishment of
hierarchies may have been initiated indicated by the progressive increase in biting behaviour (Braddock, 1945; Scott and Currie, 1980; Noble et al., 2012). Continued aggression beyond the initial hierarchy establishment could be detrimental causing chronic stress in some individuals (Sloman and Armstrong, 2002), therefore practices promoting more rapid and peaceful hierarchy establishment would improve the welfare of ornamental fish following transport.

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

Conclusion

This study is the first to record water quality and ornamental fish welfare through a commercial chain. This study emphasises the complexity of studying ornamental fish welfare during commercial transport and that exposure to changes in water chemistry, within acceptable water quality parameters, should also be considered in studies of fish transport as water quality was found to influence the recorded behaviours. The study of animals within commercial transport chains often requires non-
invasive methods; here we find that monitoring behaviour is a useful tool but one that is species-specific.

Acknowledgements

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6. Declaration of interest

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

Figure 1: (A) DO levels (%) \((n=10)\) and (B) temperature (°C) \((n=10)\) through the transport stages (WS A=wholesaler arrival, WS 1= wholesaler recovery day 1, WS 2= wholesaler recovery day 2, WS 5= wholesaler recovery day 5, P= packing for transport to stores, Store A= store arrival, Store 1= store recovery day 1, Store 2= store recovery day 2, Store 5= store recovery day 5) showing the mean (diamond), median, upper and lower 25th percentile, and outliers. Letters indicate differences between species within a specific time point where boxes sharing a letter are not significantly different.

Asterisks indicate significant differences within a species between different transport stages (post-hoc Tukey, \(P<0.05\)).

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

Figure 3: (A) \(\text{TAN}\) (ppm) \((n=10)\) and (B) \(\text{NH}_3\) \((n=10)\) through the transport stages (WS A=wholesaler arrival, WS 1= wholesaler recovery day 1, WS 2= wholesaler recovery day 2, WS 5= wholesaler recovery day 5, P= packing for transport to stores, Store A= store arrival, Store 1= store recovery day 1, Store 2= store recovery day 2, Store 5= store recovery day 5) showing the mean (diamond), median, upper and lower 25th percentile, and outliers. Letters indicate differences between species within a specific time point where boxes sharing a letter are not significantly different. Asterisks indicate significant differences within a species between different transport stages (post-hoc Tukey, \(P<0.05\)).

Figure 4: (A) \(\text{NO}_3^-\) \((n=10)\), (B) \(\text{SO}_4^{2-}\) \((n=10)\), (C) \(\text{Cl}^-\) \((n=10)\) and (D) \(\text{PO}_4^{3-}\) \((n=30)\) through the transport stages (WS A=wholesaler arrival, WS 1= wholesaler recovery day 1, WS 2= wholesaler recovery day 2, WS 5= wholesaler recovery day 5, P= packing for transport to stores, Store A= store arrival, Store 1= store recovery day 1, Store 2= store recovery day 2, Store 5= store recovery day 5) showing the mean (diamond), median, upper and lower 25th percentile, and outliers. (A-C) Letters indicate differences between species within a specific time point where boxes sharing a letter are not significantly different. Asterisks indicate significant differences within a species between different transport stages. (D) Letters indicate differences between time points where boxes sharing a letter are not significantly different (post-hoc Tukey, \(P<0.05\)).

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

Figure 6: Presence of (A) *Aeromonas* sp. (%), (B) *Pseudomonas* sp. (%), (C) *Mycobacterium* sp. (%) through the transport, \(n=30\). Letters indicate significant difference between transport stages where bars sharing a letter are not significantly different (post-hoc Tukey, \(P<0.05\)).
Figure 7: Neon tetra behaviour, (A) crowding behaviour (%), (B) occurrence of group formation and (WS A=wholesaler arrival, WS 1= wholesaler recovery day 1, WS 2= wholesaler recovery day 2, WS 5= wholesaler recovery day 5, P= packing for transport to stores, Store A= store arrival, Store 1= store recovery day 1, Store 2= store recovery day 2, Store 5= store recovery day 5) showing the mean (diamond), median, upper and lower 25th percentile, and outliers, n=10. Letters indicate differences between time points where boxes sharing a letter are not significantly different (post-hoc Tukey, P<0.05).

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

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

Figure 10: Oranda eye colour by the presence of Mycobacterium sp. showing the mean (diamond), median, upper and lower 25th percentile, and outliers, n=10. Asterisks indicate difference (post-hoc Tukey, P<0.05).
Figure 1

(A) Oxygen (%)

(B) Temperature (°C)

Species
- Neon tetra
- Oranda
- Variatus platy

Stage of Transport
- WS A
- WS 1
- WS 2
- WS 5
- P
- Store A
- Store 1
- Store 2
- Store 5
Figure 2

(A) pH

Species
- Neon tetra
- Oranda
- Variatus platy

(B) Carbonated Hardness (°dKH)

Stage of Transport

WS A, WS 1, WS 2, WS 5, P, Store A, Store 1, Store 2, Store 5

Letters indicate significant differences among groups.
Figure 3

(A) Total Ammonia Nitrogen (ppm)

(B) Unionised Ammonia (ppm)

Stage of Transport

Species

- Neon tetra
- Oranda
- Variatus platy
Figure 6

(A) Aeromonas sp. Present (%)

(B) Pseudomonas sp. Present (%)

(C) Mycobacterium sp. Present (%)
Figure 8

Erratic Swimming (30 min⁻¹)

Stage of Transport

WS A
WS 1
WS 2
WS 5
P
Store A
Store 1
Store 2
Store 5

ab
ab
ab
ab
ab
a
ab
b
**Supplementary Material**

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

<table>
<thead>
<tr>
<th></th>
<th>Transport Stage</th>
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<tbody>
<tr>
<td><strong>Neon tetra</strong></td>
<td></td>
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<tr>
<td>Mortality</td>
<td>$X^2=54.667$, df=48, p= 0.236</td>
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<tr>
<td>Ventilation rate</td>
<td>Not visible</td>
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<tr>
<td>Body condition</td>
<td>No occurrence</td>
</tr>
<tr>
<td>Eye colour</td>
<td>Not visible</td>
</tr>
<tr>
<td>Water cortisol concentrations</td>
<td>$F_{1,4}=0.251$, p= 0.642</td>
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<tr>
<td>Crowding</td>
<td>$F_{6,44}=0.028$, p=0.999</td>
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<tr>
<td>Gasping</td>
<td>No occurrence</td>
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<tr>
<td>Erratic swimming</td>
<td>Not visible</td>
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<tr>
<td>Freezing</td>
<td>Not visible</td>
</tr>
<tr>
<td>Biting</td>
<td>Not visible</td>
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<tr>
<td><strong>Oranda</strong></td>
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<tr>
<td>Mortality</td>
<td>$X^2=16.029$, df=16, p= 0.451</td>
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<tr>
<td>Ventilation rate</td>
<td>$F_{7,41}=1.003$, p= 0.612</td>
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<td>Body condition</td>
<td>$F_{8,62}=1$, p= 0.612</td>
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<tr>
<td>Eye colour</td>
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<tr>
<td>Water cortisol concentrations</td>
<td>$F_{1,4}=1.198$, p= 0.612</td>
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<td>Behavior</td>
<td>Statistic</td>
</tr>
<tr>
<td>--------------------------</td>
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<tr>
<td>Latency to feed</td>
<td>$F_{1,5}=0.511$, $p=0.765$</td>
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<tr>
<td>Freezing</td>
<td>No occurrence</td>
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<tr>
<td>Biting</td>
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<tr>
<td>Variatus platy Mortality</td>
<td>$X^2=63.358$, $df=64$, $p=0.499$</td>
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<tr>
<td>Ventilation rate</td>
<td>$F_{7,41}=1.003$, $p=0.612$</td>
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<td>Body condition</td>
<td>$F_{8,62}=1$, $p=0.816$</td>
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<td>Eye colour</td>
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<tr>
<td>Water cortisol concentrations</td>
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</tr>
<tr>
<td>Gasping</td>
<td>No occurrence</td>
</tr>
</tbody>
</table>