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The Use of Feed and Water Additives for Live Fish Transport

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Abstract

The transport of live fish for aquaculture, either for food or as companion animals, presents a major issue for animal welfare. The stressors associated with live transportation are well documented with a focus on maintaining water quality during transport to reduce stress. Far less considered is our ability to enhance health and welfare during transport through the use of dietary and water additives prior to and during transport. With increasing interest in the use of plant essential oils as feed additives in food fish aquaculture and the increased availability of products claiming to alleviate stress in ornamental species, there is a need for scientific investigation into these potential welfare-promoting methods. Here we summarise current knowledge on the use of food additives, water conditioners, antibiotics, antimicrobials, and probiotics to promote fish health during transport. This review aims to highlight the gaps in our knowledge surrounding promising ways of promoting fish health during transport and to stimulate new research in this area.

Key Words: fish transport, stress, water quality, welfare management

Introduction

Fish production as a food source was valued at US$160 billion in 2014 (FAO 2016) and the estimated worth of the ornamental fish industry (including wholesale, wages, retail sales, associated materials etc.) is approximately US$15 billion (FAO 2011). In both aquaculture enterprises, fishes will be transported at some point in the production chain although the duration and the type of transport varies greatly between the two industries. Fishes destined for food are often transported by road to various facilities in open systems where continuous monitoring of water quality and access to fish during transport is possible (Van de Sande 1974; Berka 1986; Rimmer & Franklin 1997; Lekang 2007; Espinosa-Curiel *et al*. 2016). In contrast, ornamental fishes tend to be transported over greater distances in closed systems (e.g. in plastic bags shipped by aeroplane) (Braker 1974; Berka 1986; Swann 1992; Rimmer & Franklin 1997; Cole *et al*. 1999; Marine Aquarium Council 2001; Lim *et al*. 2007). While fish transport practices have been reviewed extensively, exact information on mortality during transport is particularly limited for the ornamental fish trade with estimated values ranging from a few per cent to greater than 80% (Rubec & Cruz 2005). High mortality is also reported during recovery from transport (Froese 1988; Sadovy 2002; Rubec & Cruz 2005).

Despite the differences in transport practice between food fishes and fishes destined for the pet industry, welfare challenges for transportation are very similar. Stressors encountered during transport include handling prior to transport, deterioration of water quality during transport and increased susceptibility to metabolic shock, stress, infection and disease after transport (Pickering *et al*. 1982; Portz *et al*. 2006; Sampaio & Freire 2016). There are several excellent reviews on how water quality and husbandry practices can

improve welfare (Berka 1986; Swann 1992; Cole *et al.* 1999; Crosby *et al*. 2005a, b; Lim *et al.* 2007; Harmon 2009), but far less scientific attention has focused on the potential to enhance welfare during transport over and above good husbandry practice. For example, a range of commercial and non-commercial products have been suggested to aid fish health during transport but there is a considerable absence of studies within the scientific literature surrounding these products. This review will firstly consider whether the stress of transport can be alleviated by feed or water treatment prior to transport, and secondly consider current knowledge surrounding water additives during transport.

Can we Prepare Fishes for Transportation Stress?

Dietary supplements are commonly used in aquaculture for a wide variety of reasons including stress reduction (Peng *et al*. 2013; Vallejos-Vidal *et al*. 2016), improving specific and non-specific immune resistance (Wang *et al*. 2006; Vallejos-Vidal *et al*. 2016), enhancing colouration (Pan & Chien 2009; Kouba *et al*. 2013) and increasing growth rate (Vallejos-Vidal *et al*. 2016). Therefore, prior to transport, there is the opportunity to use dietary supplements to enhance the immune system and improve stress tolerance (Volpatti *et al*. 1998; Lim *et al*. 2002; Rollo *et al*. 2006). Dietary supplements that have been tested in relation to transport in fishes include glucan, probiotics, ascorbic acid, carotenoids and herbal supplements.

Glucan is a polysaccharide which enhances non-specific immunity in fishes and reduces susceptibility to stress and the immunosuppressive effects of stress (Volpatti *et al*. 1998; Kim *et al*. 1999; Vallejos-Vidal *et al*. 2016). Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) fed a diet with no (0%), low (0.1%), medium (0.5%) or high (1%) levels of

glucan for four weeks showed differences in non-specific immune responses to 2 h simulated transport stress (Volpatti *et al*. 1998). Following transport stress, lymphocytes decreased in the group fed 0% glucan, whereas in all the glucan-fed groups, lymphocytes, monocytes and neutrophils, increased demonstrating that glucan increases the non-specific immune response. Phagocytosis and respiration burst activity of cells were also higher in glucan-fed groups than in the 0% group. A sub-group of fish was fed the experimental diets for a further two months following transport to determine the long-term effect of glucan on the digestive tract. Prolonged exposure to glucan resulted in slight deterioration within the epithelial cells of the stomach and gut (Volpatti *et al.*, 1998) suggesting that prolonged ingestion of glucan may not be beneficial.

Probiotics are either a mono or mixed culture of live micro-organisms that are ingested and multiply in the gut of host organisms in order to improve the indigenous microflora (Havenaar *et al*. 1992; Cross, 2002). Probiotics are increasingly being considered as a safer and more environmentally-friendly alternative to antibiotics with their use in aquaculture increasing as a response to public demand for antibiotic-free fishes (Martínez Cruz *et al*. 2012). The desired effects of probiotics include improved larval development, growth promotion, stimulation of the immune system, pathogen and disease control, stress resistance and improved water quality (Martínez Cruz *et al*. 2012). Ambas *et al*. (2015) simulated transport of marron (*Cherax cainii*, Austin 2002) reared on marron commercial feed as a control, or control feed enriched with *Bacillus mycoides* (Flugge 1886). After 48 h, survival of marron fed the control diet was 93.3 ± 2.8% S.E. vs. 100% in the probiotic group. Marron in the probiotic group also had a higher intestinal bacterial population and total haemocyte count than the controls, indicating an improved immune status when fed probiotics. Although studies on the direct benefits of probiotics in alleviating transport stress

are limited, the advantages of probiotics in response to stressors relevant to transport have also been demonstrated. Rollo *et al.* (2006) reared sea bream (*Sparus aurata,* Linnaeus 1758) larvae with a mixture of *Lactobacillus fructivorans* (Charlton *et al.* 1934) and *Lactobacillus plantarum* (Orla-Jensen 1919) for either 20 or 42 days using rotifers (*Brachionus plicatilis,* Müller 1786) and/or *Artemia salina* (Linnaeus 1758) as a vector. At the end of the rearing period, 600 fry were exposed to 6.3 pH for 1 h. The probiotic group had lower levels of mortality and whole body cortisol. Hsp70 gene expression increased after exposure to low pH with the greatest increase in Hsp70 found in sea bream reared with probiotics.

Fishes cannot synthesise ascorbic acid (vitamin C) and rely on absorption through their food (Sales & Janssens 2003; Peng et al. 2013). Supplementing feed with ascorbic acid has been found to reduce mortality following a stressor (Lim *et al*. 2002). Peng *et al*. (2013) fed silver pomfret (*Pampus argenteus,* Euphrasén, 1788) a diet supplemented with ascorbic acid (L-ascorbyl- 2-polyphosphate, 35% ascorbic acid equivalent at a concentration of 100, 450 or 800 mg ascorbic acid kg^{-1} diet) for 9 weeks. Silver pomfret were then transported in darkened plastic bags for 4 h. Diet supplementation with ascorbic acid significantly reduced serum cortisol and glucose levels as well as mortality, indicating that high ascorbic acid successfully reduced stress associated with transport.

Abreu *et al.* (2014) fed wild-caught pencilfish (*Nannostomus trifasciatus*) one of four experimental diets with 0%, 0.01%, 0.1% or 0.5% beta 1,3 glucan added for 7 days. The fish were then fasted and transported for 24 h. The addition of beta 1,3 glucan reduced the net loss of K^+ during the first 3 h of transport. The addition of 0.5% beta 1,3 glucan to the feed increased the net influx of Na^+ between 3 and 12 hours of transport.

Combinations of ascorbic acid and glucan as supplements have been trialled in relation to transport stress. Barros *et al*. (2014) supplemented the diet of Nile tilapia (*Oreochromis niloticus*, Linnaeus 1758) with a basal diet of 125 mg kg^{-1} of ascorbic acid (BD) followed by a diet containing 0.1% ß -glucan and 600 mg kg^{-1} ascorbic acid (GD). The fish were reared on BD for 20 days, after this period the fish were fed GD for variable durations (7, 15, 30, or 45 days) prior to undergoing transport stress. Fish from all four treatments were transported for 4 h in 100 l net cages within a 600 l fish transport tank. During recovery from the transport, fish fed 0.1% ß -glucan and 600 mg kg^{-1} ascorbic acid for 7 days had the highest cortisol concentrations and greatest increase in red blood cells and haemoglobin. However, longer exposure to ß-glucan and ascorbic acid reduced plasma cortisol and after 72 h, cortisol had returned to baseline in Nile tilapia fed this diet for longer than 7 days. As ß-glucan and high levels of ascorbic acid were not tested separately, it is unclear which supplement provided the observed improvements or whether it was the two combined.

Organisms undergoing stress can experience a shortage of oxygen at the cellular level causing abnormal oxidative reactions in the aerobic metabolic pathway and the generation of reactive oxygen species (ROS) (Rånby & Rabek 1978; Pan *et al*. 2010) which if not inactivated can cause oxidative damage to lipids, proteins, carbohydrates and nucleotides (Yu 1994; Chew 1995; Halliwell & Gutteridge 2015). Carotenoids are pigment molecules with known antioxidant properties that are important to animal health by inactivating free radicals. Fishes cannot synthesise carotenoids *de novo* and must acquire them through their diet. In food fish aquaculture, diets are often supplemented with carotenoids to improve fillet colouration and to reduce oxidative stress. The use of carotenoids, in particular astaxanthin, as a dietary supplement in aquaculture has been extensively reviewed and found to improve

159 stress resistance (Johnson *et al*. 1980; Lorenz & Cysewski 2000; Pan *et al*. 2003, 2010;
160 Higuera-Ciapara *et al*. 2006; Pan & Chein 2009; Jagruthi *et al*. 2014; Lim *et al*. 2017).
161 Dietary carotenoids such as astaxanthin and ß-carotene have been shown to help fish cope
162 with stressors that may act as components of transport stress (e.g. hypoxia, Pan *et al*. 2010;
163 high ammonia, Pan *et al*. 2011). Therefore, the potential benefits of carotenoids as dietary
164 supplements to prepare fish for transport stress warrants future study.
165
166 In aquaculture, plant extracts are mostly used as feed supplements, although some may
167 be added directly to the water (see **Additives During Transport** below). In 2011 the total
168 global herbal drug market was estimated at US $62 billion and is predicted to grow to US $5
169 trillion by 2050 with an annual growth rate of 5-15% (Harikrishnan *et al*. 2011). Plant-
170 derived products can be effective as antioxidants, growth promoters, appetite stimulators,
171 immune stimulants and stress reducers along with having anti-inflammatory and anti-
172 carcinogenic properties (Citarasu 2010; Harikrishnan *et al.* 2011; Merlini *et al*. 2014; Bulfon
173 *et al*. 2015). The use of plant essential oils as dietary supplements in aquaculture was recently
174 reviewed (Sutili *et al*. 2017) but to our knowledge only two studies have considered the
175 effects of plant extracts on stress tolerance during transportation through administration as a
176 dietary supplement. Turmeric (*Curcuma longa,* Linnaeus 1758) has a variety of documented
177 medicinal properties (anti-inflammatory, Araújo & Leon 2001; immunostimulant,
178 Chattopadhyay *et al*. 2004; antioxidant, Luthra *et al*. 2001, Saccol *et al*. 2016; anaesthetic,
179 Saccol *et al*. 2016; anti-microbial, Luthra *et al*. 2001; anti-parasitic, Araújo & Leon 2001).
180 Supplementing the diet of juvenile yellow tail tetra (*Astyanax aff. bimaculatu,* Linnaeus
181 1758) with turmeric for 60 days before a simulated 24 h transport reduced mortality, plasma
182 lactate and plasma glucose concentrations compared to controls (Ferreira *et al.*, 2017). *Aloe*
183 *vera* (Burman 1768) is commonly used in humans for a wide range of medicinal properties

(*e.g.* Vázquez *et al*. 1996; Reynolds & Dweck 1999; Vogler & Ernst 1999; Choi *et al*. 2001; Choi & Chung 2003; Mahor & Ali 2016) but little is known about the benefits of using *A. vera* as a dietary supplement in aquaculture. Zanuzzo *et al*. (2017) administered one of four diets (0%, 0.5%, 1% or 2% *A. vera*) for 10 days prior to a 4 h transport of juvenile pacu (*Piaractus mesopotamicus*, Holmberg 1887). Immediately following transport, the pacu were divided into three sub-groups: a non-injected control group; a buffer injected group and a group injected with inactivated *Aeromonas hydrophila* (Chester 1901) to stimulate their immune system. This is particularly relevant to transport stress studies as fishes can become highly susceptible to bacterial and viral infections during transport (Yanong 2003; Crosby *et al*. 2005b). On arrival and after 24 h recovery, non-injected *A. vera* fed groups had significantly higher cortisol concentrations than the non-injected control fish. However, in the *A. hydrophila* injected fish, the cortisol levels of the fish fed 1% *A. vera* were significantly lower than in the other groups. Immediately after transport, leukocyte respiratory burst was higher in all (injected and non-injected) *A. vera* fed fish than in the control fish but no significant difference was found between the treatments after 24 h of recovery. The haemolytic activity of the complement system was significantly higher in the *A. vera* fed fish on arrival and after recovery than in the control fish. The results indicate that *A. vera* improved the immune system of juvenile pacu after transport and improved the stress recovery of infected individuals. No explanation for why *A. vera* slowed recovery rate of the non-injected juvenile pacu yet improved the recovery rate of the injected group was given. Further research into the use of herbal dietary supplements in relation to transport of fishes is clearly needed.

The Use of Additives During Transport

There are many reviews that have considered the importance of good water quality during live fish transport (Berka 1986; Swann 1992; Cole *et al.* 1999; *Crosby et al*. 2005a, 2005b; Lim *et al.* 2007; Harmon 2009) and a variety of products can be added to the water to maintain water quality or alleviate the problems of waste products (e.g. pH buffers, zeolites, AmQuel®, nitrifying bacteria). Even when water quality is optimal for the duration of transport, there are many other stressful factors (e.g. handling, high loading density and crowding) (Pickering *et al*. 1982; Portz *et al*. 2006; Sampaio & Freire 2016); far less research has focused on whether it is possible to add compounds to the water to alleviate the physiological effects of stress. As demonstrated above, the benefits of dietary supplements for improving health and welfare during transport may require many weeks of preparation; the addition of compounds to the water for the period of transportation requires far less advance planning. Some products such as water conditioners and salt are added directly to the transport water along with fishes while others may require exposure for a short time immediately prior to transport (e.g. anaesthetics). The promotion of physiological well-being through addition of compounds to the water can occur *via* different physiological mechanisms such as sedation, protection of mucus integrity and disease prevention. While the use of traditional water additives such as salt and synthetic anaesthetics have been well explored, the use of plant extracts as water conditioners and antimicrobial agents has received less attention. Here we will briefly discuss the more traditional water additives but will focus primarily on the use of novel and emerging water additives.

Managing Stress by Sedation

231

232 Anaesthetics are one of the most commonly used additives in the transport of fish (Lim *et*
233 *al*. 2003; Harmon 2009; Cupp *et al*. 2017). Anaesthesia is defined as *"a state caused by an*
234 *applied external agent resulting in a loss of sensation through depression of the nervous*
235 *system"* (Akerman *et al.* 2005). Sedation can lower a fish's metabolic rate (Ross & Ross
236 2008) resulting in improved water quality (Pattanasiri *et al*. 2016), lower levels of stress (Ims
237 2011) and often allows transportation at higher loading densities (Cupp *et al*. 2017).
238 Frequently used synthetic anaesthetics, such as MS-222 (ethyl 3-aminobenzoate
239 methanesulfate) and benzocaine, have been widely reviewed elsewhere (Ross *et al*. 2008;
240 Carter *et al*. 2011; Javahery *et al*. 2012; Readman *et al.* 2013; Husen & Sharma 2014) and
241 will not be discussed in detail here. Generally, synthetic anaesthetics initially induce stress
242 before having a delayed stress-reduction effect (Ims 2011; Readman *et al*. 2013). An
243 alternative to sedation through synthetic compounds is to use natural compounds such as
244 essential oils.
245

246 The most commonly used essential oil is clove oil (*Syzygium aromaticum,* Linnaeus
247 1758). Clove oil has been extensively reviewed for its use as an anaesthetic (e.g. Javahery *et*
248 *al*. 2012) but many other less researched essential oils have been considered for use during
249 fish transport. Most studies have considered the use of essential oils during transport by
250 adding them to the water at set concentrations. *Condalia buxifolia* (Reisseck 1861)
251 methanolic extract induced sedation in silver catfish (*Rhamdia quelen*) for 6 h and during
252 transport improved survival, water quality and reduced ion loss (Becker *et al*. 2013). Salbego
253 *et al*. (2015) also found similar results when they sedated non-starved silver catfish with
254 methanolic extract of *C. buxifolia* prior to transport and added *C. buxifolia* solution to the
255 transport water. Addition of *C. buxifolia* improved water quality, reduced total ammonia

256 nitrogen (TAN) levels, slowed metabolism and reduced net ion efflux. Lipoperoxidation and
257 carbonylation of proteins decreased in silver catfish transported in *C. buxifolia*,
258 demonstrating less ROS production. Low concentrations (0.5-10 µl l^{-1}) of *C. buxifolia*
259 induced fast sedation and higher doses did not cause harmful effects suggesting it is safe to
260 use to induce sedation (Becker *et al*. 2013).
261
262 The use of essential oils in the water is summarised in Table 1. Interestingly, Pattanasiri
263 *et al*. (2016) tested the release rate of low-density polyethylene (LDPE) bags coated with
264 clove oil over a 48 h period. After the initial 2 h, the release rate of clove oil was almost
265 constant at 12 mg l^{-1} when the bag contained 75 ml water and 14 mg l^{-1} when the bag
266 contained 150 ml water. These levels induced sedation in Siamese fighting fish (*Betta*
267 *splendens*, Regan 1910) but not anaesthesia. Survival of Siamese fighting fish transported in
268 the clove oil-coated bags was significantly higher, with lower ammonia concentrations and
269 higher dissolved oxygen than in control un-coated bags. Siamese fighting fish did not appear
270 to experience any detrimental effects of prolonged clove oil exposure.
271
272 In summary, several essential oils are effective in inducing sedation in fish, however,
273 most essential oils have only been tested at one or a few concentrations and in a single
274 species; few have been tested for use in fish transport. The mechanism of action can vary
275 between essential oils and in the most part is poorly understood. It is known that different
276 species have different aversive reactions to anaesthetics, so there is a need to further explore
277 the use of essential oils on individual species of interest (Javahery *et al*. 2012; Husen &
278 Sharma 2014; Chambel *et al*. 2015). With the increased commercialisation of essential oils,
279 or components of, for use in fish husbandry (e.g. AQUI-S; http://aquatactics.com/aqui-s-

20e/), the potential for better, more refined chemicals for improving fish welfare during transport compared to more traditional synthetic chemicals seems likely.

Maintaining Mucus Integrity

The epidermal layer of fishes excretes protective mucus that serves as a barrier to the external environment (Harnish *et al*. 2011; Ottesen & Olafsen 1997; Shephard 1994). During transport and handling, fishes can lose their protective mucus, resulting in an increased risk of injuries (Harnish *et al*. 2011). Mucus loss can result in disturbed osmoregulation, loss of scales, skin damage, and bacterial, fungal and parasitic diseases (Wedemeyer 1996). When fishes are stressed or transported in large numbers within a single bag, the risks of losing mucus increases as fish are more likely to come into contact with the bag or other individuals. Protecting epidermal mucus can result in a higher quality of fish at arrival in terms of physical health, reduced risk of infection and improved aesthetic appearance.

Water conditioners usually refer to compounds added directly to the transport water of fish to reduce stress by means other than sedation. Water conditions may be either pure herbal extracts or combined with other products into a commercially available product. A review on the use of polymer-based water conditioners to reduce handling-related injuries (Harnish *et al*. 2011) identified three studies (summarised in Table 2). Wedemeyer (1996) presented findings of user surveys and husbandry procedures finding that polymer-based water conditioners reduce mortality.

Stress Coat® (API Aquarium Pharmaceuticals Inc., n.d.) is a commercially available water conditioner which is recommended for addition to water during transport and to tank water during other potentially stressful procedures. Several studies have used Stress Coat® in

husbandry procedures (Earley *et al*. 2006; Colburn *et al*. 2008; Harnish *et al*. 2011; Wong *et al*. 2015), however, much of the information on Stress Coat® is not in the peer-reviewed literature. Snellgrove *et al.* (2007) found that water cortisol levels excreted by goldfish exposed to Stress Coat® were lower following netting compared to goldfish netted with no Stress Coat® exposure. Edmonds (2016) found that Stress Coat® exposure during transport did not reduce excretion of cortisol but reduced conspecific aggression in guppies post-transport.

The main components of Stress Coat® (manufactured by Mars Fishcare) are *Aloe barbadensis* Mill (also known as *A. vera*) (1-10%), water (>80%), polyvinylpyrrolidone (PVP) and other non-hazardous ingredients (trade protected) (1-10%) (Mars Fishcare Inc. 2014). Most of the limited peer-reviewed research carried out directly on *A. vera* focus on it as a dietary supplement used to alleviate stressors not relevant to the transport of fish (*e.g.* Dotta *et al*. 2014; Gabriel *et al*. 2015a,b; Kim *et al*. 1999; Taiwo *et al*. 2005; Zanuzzo *et al*. 2015a,b). One study looked at *A. vera* as a dietary supplement prior to transport (Zanuzzo *et al*. 2017; see above) and an additional study by the same group used *A. vera* in the water during transport of fish. Zanuzzo *et al*. (2012) dissolved *A. vera* powder (concentrations: 0, 0.02, 0.2 and 2 mg l^{-1}) in the transport water of matrinxã (*Brycon amazonicus*, Spix and Agassiz 1829). *Aloe vera* increased the activity of the immune system, by enhancing the respiratory activity of leukocytes, in matrinxã following handling but the effects of *A. vera* were no longer apparent at the end of the 4 h transport. *Aloe vera* has potential to improve the condition of fish during transport procedures but more research is needed to gain a better understanding of the possible benefits and the best method of administration.

Sung *et al*. (2012) tested another patented product Pro-Tex® (Bradan Limited, a soluble variant of TEX-OE®) to determine whether it improved the resistance of juvenile carp

329 (*Cyprinus carpio,* Linnaeus 1758) to high ammonia levels. Pro-Tex® contains an extract of
330 the prickly pear cactus (*Opuntia ficus indica*, (L.) Miller 1925) which increases heat-shock
331 protein expression in humans and fishes (Wiese *et al*. 2004; Roberts *et al*. 2010; Sandilands
332 *et al*. 2010). Exposure of carp to Pro-Tex® (2 µl 50 l^{-1} water) for 2 h increased survival from
333 50 to 95% and 0 to 20% when exposed to 5.92 mg l^{-1} and 14.21 mg L^{-1} of NH_3 respectively
334 for 1 h. Sung *et al*. (2012) also found that Pro-Tex® increased expression of heat shock
335 protein (Hsp70) in gill and muscle tissue of carp which may suggest potential benefits of Pro-
336 TEX® for fish transport. Hales *et al*. (1990) evaluated the injury-preventing capacity of a
337 water-soluble gel-coating (composed of antibiotics and non-disclosed pharmaceutical
338 components) applied to the hands of fish handlers. The unexpected results showed that spot
339 croaker (*Leiostomus xanthurus,* Lacepède 1802) handled with gel-coating had a higher
340 mortality than fish handled by collectors with non-coated hands. The authors hypothesised
341 that the composition of the gel was different from that of the mucus of the fish as mucus
342 mostly consists of protein along with lipids, carbohydrates and nucleic acids (Al-Hassan *et al*.
343 1982). Austin *et al.* (2009), published a non-peer reviewed report about the use of
344 ULTIMATE® (AquaScience Technologies) in the transport water of koi carp. ULTIMATE®
345 has two main components, the ingredients for ClorAm-X® (the original AmQuel®)
346 (composed of sodium hydroxymethanesulfonate, AquaScience Technologies) and the
347 ingredients for Stress-X® (the original NovAqua®) (water, sodium thiosulfate, buffers,
348 electrolytes, proprietary synthetic polymer formulation and preservatives, Aquarium
349 Solutions). In addition, ULTIMATE® contains a dechloraminating agent, electrolytes
350 (including calcium, sodium and chloride ions), a polymer system and product stabilizers
351 (Austin *et al*. 2009). After 8 h of simulated sealed transport, the koi transported in
352 ULTIMATE® had shorter recovery times than the control fish indicated by accelerated
353 reduction in mucosal levels of haemoglobin. Unfortunately, sample size was not sufficient for

definitive results, and in addition, the methods used to detect levels of haemoglobin in the mucus were not precise enough. No published peer-reviewed paper was found that used ULTIMATE® in fish transport, however it is an area worth investigating based on the observations made by Austin *et al*. (2009). Despite many studies using substances such as Stress Coat®, Novaqua® and Polyaqua®, little peer-reviewed information is available on the efficacy of these substances, particularly in maintaining mucus integrity.

Disruption of epidermal mucus can cause many detrimental effects including disruption to osmoregulation. The stress of transport itself can also cause changes in osmoregulation (Barton & Iwama 1991; Baldisserotto *et al*. 2007) and so during transport of freshwater species, salt (NaCl) can be added to the water to reduce the difference between the internal osmolality of the fish and that of its environment thereby reducing physiological workload required to maintain homeostasis (Nikinmaa *et al*. 1983). Table 3 gives examples of studies that have investigated the effects of NaCl addition during transport. It is clear that for some species, the addition of NaCl during transport may be beneficial but given the range of salinities that different species inhabit and differences in osmoregulatory capacity from stenohaline to euryhaline, the use of NaCl as an additive will always be very species and life-stage specific. Additionally, Tacchi *et al*. (2015) compared the skin morphology of non-transported rainbow trout to that of rainbow trout transported in fresh or salt water (5 g NaCl l^{-1}) using electron microscopy. Fish transported in salt water had a thin layer of mucus whereas fish transported in fresh water had a thick deposit of mucus. It was suggested that the addition of NaCl slowed down the release of mucus from goblet cells. A ~50-fold increase in skin-associated bacteria in fish transported in fresh water was seen compared to a ~10-fold increase in the salt water group (Tacchi *et al*. 2015). While salt water transported fish had a thinner mucus layer, the mucus layer of these fish appeared to be in better condition showing

379 that NaCl also has the potential to reduce subsequent stress caused by bacteria and improve
380 skin mucus condition.
381
382 Prevention of Stress-Related Diseases
383
384 When fishes experience high levels of stress they become more susceptible to bacterial
385 diseases, which can result in higher mortality (Yanong 2003, Crosby *et al*. 2005b). To
386 prevent proliferation of bacteria in the transport water while the fish's immune system is
387 weakened, antibiotics are sometimes used. Amend *et al*. (1982) tested the efficacy of several
388 antibiotics (kanamycin, gentamicin, chloramphenical, streptomycin, neomycin and
389 furazolidone, each at 20 mg l^{-1}), the antiseptic acriflavine (10, 20, and 100 mg l^{-1}), the
390 disinfectant chlorine dioxide at 20 mg l^{-1}, and the antimicrobial methylene blue (10 and 100
391 mg l^{-1}). The tests were done by simulating 48 h transport with southern platy (*Xiphophorus*
392 *maculatus*, Günther 1866) and checking the efficacy of each substance to control bacteria
393 levels. Kanamycin and gentamicin were found to be toxic to the fish at these concentrations.
394 Methylene blue, chlorine dioxide, furazolidone, and acriflavine did not prevent bacteria
395 growth. Acrafiavine at 100 mg l^{-1}, chloramphicol and streptomycin controlled bacteria levels,
396 but caused mortality in the fish. Only neomycin was effective against bacteria and safe for
397 the fish. Antibiotic resistance in ornamental fishes has been studied since the late 1970s and
398 the literature shows that resistance emerges when new antibiotics become widely available
399 (del Rio-Rodriguez & Turnbull 2002; Rose *et al*. 2013; Trust & Whitby 1976; Verner-
400 Jeffreys *et al*. 2009). A study on ornamental fishes by Rose *et al*. (2013) found that the most
401 effective antibiotics (cefotaxime and kanamycin) were effective against only 45% and 44%
402 of their target bacteria respectively, and of these, 16% and 35% had developed resistance. In
403 this study, nine bacteria were resistant to all the tested antibiotics, only one showed no

404 resistance at all. Dixon *et al*. (1999) found similar results in a study looking at bacterial
405 resistance in fish imported from Singapore. Over 50% of the bacteria isolated were resistant
406 to 7 out of 12 tested antibiotics. In addition to increased resistance, antibiotics can cause
407 increased levels of stress in fishes. Cururu stingray (*Potamotrygon cf histrix*, Müller and
408 Henle 1841) transported in water containing tetracycline (200 mg l^{-1}) for 24 h had elevated
409 corticosterone levels after 12 h compared to controls (Brinn *et al*. 2012). The administration
410 of antibiotics during transport is also problematic because the use of antibiotics is strictly
411 monitored and regulated in many countries (Cole *et al*. 1999; Crosby *et al*. 2005b; Brinn *et*
412 *al*. 2012).
413
414 Probiotics can be added to transport water to improve water quality and reduce stress
415 arising from low quality water. Efinol®L is a commercial probiotic product containing
416 *Bacillus subtilis* (Ehrenberg 1835), *Bacillus licheniformis* (Weigmann 1898), *Lactobacillus*
417 *acidophilus* (Moro 1900) and *Saccharomyces cerevisiae* (Hansen 1883); it also comprises
418 amino acids, vitamins, minerals, free-flow and anti-caking agents (calcium carbonate and
419 silica). Marbled hatchetfish (*Carnegiella strigata*, Günther 1864) were transported in a water
420 solution containing 10 mg l^{-1} probiotic Efinol®L. After 24 h of transport, water containing the
421 probiotic solution had higher dissolved oxygen levels and lower ammonia concentrations
422 (Gomes *et al.* 2008). Fish in the control group had higher body cortisol levels and higher
423 efflux of Na^+ and K^+. The reduced stress levels seen in marbled hatchetfish treated with
424 probiotics could be attributed to either a direct effect of Efinol®L on physiology or an indirect
425 effect of improved water quality. Using a similar protocol, Gomes *et al*. (2009) transported
426 cardinal tetra (*Paracheirodon axelrodi*, Schultz 1956) in water containing Efinol®L. The
427 addition of Efinol®L resulted in a higher survival, higher water alkalinity and lower total
428 ammonia in the water. The cortisol levels of the cardinals in the Efinol®L group were

429 significantly lower after transport compared to control fish. Efinol®L is not the only probiotic
430 to have been added directly to the water. Zink *et al*. (2011) transported yellowfin tuna
431 (*Thunnus albacares*, Bonnaterre 1788) yolk sac larvae one day post-hatching for 24 h. In the
432 probiotic treatment, 300 ml of EcoAqua® (108 colony-forming units ml^{-1} in a mix of *B.*
433 *subtilis*, *B. licheniformis*, *B. megaterium* (Bary 1884), and *B. laterosporous* (Laubach 1916)
434 EcoMicrobials LLC, Miami, Florida) was added to the water. No difference in survival was
435 recorded between the treatments although the water quality of the bags containing probiotics
436 was greater than in the control bags (lower pH, lower TAN and higher dissolved oxygen).
437 Although the mechanisms of effect of these probiotic solutions are not yet fully understood,
438 and it is unclear whether there are benefits related to reduced disease susceptibility, the
439 positive effects seen in this limited number of studies suggest that more widespread
440 investigation into the addition of probiotics during transport is warranted.
441

442 **Concluding Remarks**

443

444 There is a growing market for novel compounds which can be administered either *via* the
445 diet or water to alleviate stress in fishes with the aim of increasing welfare. Perhaps driven in
446 part by an increasing desire of consumers (either of food fishes or pet fishes) to purchase
447 products which have not been exposed to synthetic chemicals, many of the emerging
448 products are based on natural compounds or enhancing natural processes. While there is
449 growing evidence that some of these compounds can improve welfare, much of the evidence
450 remains anecdotal and the mechanisms of effect have been overlooked. Dietary supplements
451 such as glucan, ascorbic acid, carotenoids, herbal supplements and probiotics may have the
452 potential to reduce stress and mortality during transport but far more research is required to
453 understand the capabilities of these supplements. Several commercially available water

conditioners have been considered in relation to transport stress, but there is a significant lack of peer-reviewed publications and publically available data on the testing of these products. Although, the process is not fully understood, probiotics can also be effective in reducing stress and mortality when added directly into transport water but as yet susceptibility to disease agents following such treatment remains unexplored. In order to enhance the welfare of fishes transported within aquaculture there is an urgent need to explore these emerging areas.

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1056 Table 1. Research into the use of essential oils during the transport of fishes.

Species	**Life stage/Size**	**Essential oil**	**Concentration**	**Stressor**	**Effect**	**Reference**
Silver catfish (*Rhamdia quelen)*	Juvenile (mean: 64.5 ± 6.1 g and 18.85 ± 0.57 cm)	*Lippia alba*	0, 10 µl l^{-1}	5, 6 or 7 h transport	Reduction of lipoperoxidation (LPO), catalase, superoxide dismutase and glutathione-S-transferase in the liver in fish transported for 5 h. Reduction of LPO in the gill of fish transported for 5 h and 7 h.	Azambuja *et al.*, 2011
Silver catfish (*Rhamdia quelen*)	mean: 262.0 ± 73.5 g, 38.5 ± 1.1 cm	*Aloysia triphylla*	0, 30, 40 µl l^{-1}	6 h transport	Lower plasma cortisol and ion loss compared to control, and higher plasma Na^+ and Cl^- concentrations. Lower hepatic glycogen and glucose	Zeppenfeld *et al.*, 2014

					concentrations in the liver. Lower muscle lactate and higher muscle glucose levels.	
Fat snook (*Centropomus parallelus*)	Juvenile (mean ± SEM: 1.6 ± 0.4 g and 4.8 ± 0.4 cm)	Menthol (5-Methyl-2-(propan-2-yl) cyclohexan-1-ol)	0, 3.7 or 7.4 mg l^{-1}	10 h transport	Effective anaesthetic for short term handling. No effects on mortality, ammonia, dissolved oxygen, nitrite levels after transport.	Sepulchro *et al.*, 2016
Fat snook (*Centropomus parallelus*)	Juvenile	*Nectandra megapotamica*	0, 15, 30 µl l^{-1}	10 h transport	Higher post-transport mortality in the 30 µl l^{-1} than the 15 µl l^{-1} and the control group.	Tondolo *et al.*, 2013
Silver catfish	mean ±	*Condalia buxifolia*	0, 5, 10 µl l^{-1}	6 h transport +	Lower water TAN in both groups	Salbego *et al*. 2015

(*Rhamdia quelen*)	SEM: 420.1 ± 8.8 g and 21.2 ± 2.3 cm			pre-sedation	transported in *C. buxifolia*.. Lower net efflux of Na^+, Cl^- and K^+ in both groups transported in *C. buxifolia*. Higher PvO_2, $PvCO_2$ and HCO_3^- in 5 µl l^{-1} group. Lower hepatic lactate in the 10 µl l^{-1} group. Lower muscle lactate in both 5 and 10 µl l^{-1} vs. control group. Improved antioxidant status.	
Silver catfish	mean ±	*L. alba*	0, 30, 40 µl l^{-1}	6 h transport +	Lower water TAN.	Becker *et al.* 2015

(*Rhamdia quelen*)	SEM: 420.1 ± 8.8 g and 21.2 ± 2.3 cm			pre-sedation	Lower net efflux of Na^+, Cl^- and K^+. Higher PvO_2, $PvCO_2$ and HCO_3^- in the 40 µl l^{-1} group. Higher plasma cortisol in the 30 µl l^{-1} compared to the control group.	
Silver catfish (*Rhamdia quelen*)	mean ± SEM: 420.1 ± 8.8 g and 21.2 ± 2.3 cm	*L. alba*	0, 30, 40 µl l^{-1}	6 h transport + pre-sedation	Lower net efflux of Na^+, Cl^- and K^+. An exposure of 30-40 µl l^{-1} induced oxidative stress and elevated cortisol.	Salbego *et al.* 2014
Silver catfish (*Rhamdia quelen*)	mean ± SEM:	*C. buxifolia*	0, 25, 50 µl l^{-1}	12 h transport	Lower non-ionized ammonia levels.	Becker *et al.* 2013

	1.50 ± 0.02 g and 165.7 ± 22.5 g				Lower net efflux of Na^+, Cl^- and K^+.	
Swordtail fish (*Xiphophorus hellerii*)	mean: 2.49 ± 0.62 g	Valerian root (*Valeriana officinalis*)	1 g l^{-1}	24 h simulated transport	Lower mortality and whole body cortisol.	Abasali & Mohamad (2010)
Nile tilapia (*Oreochromis niloticus*)	Juvenile (mean: 1.34 ± 0.07 g and 4.25 ± 0.22 cm)	*Alpinia galanga*	150 mg l^{-1}	4 h simulated transport at three loading densities (100, 200, and 300 fish/plastic bag)	No mortality. Slowed movement, higher dissolved oxygen and lower TAN in all loading densities. Lower NH_3 in the 100 and 200 fish/plastic bag densities.	Pikulkaew *et al.* (2017)
Silver catfish (*Rhamdia quelen*)	Juveniles (mean:	*Myrcia sylvatica*	25, 35 µl l^{-1}	6 h transport	Lower plasma cortisol and lactate levels, increased Na+/K+-ATPase gill activity.	Saccol *et al.* (in press)

	8.9 ± 2.7 g and 12.4 ± 1.3 cm)	Lower gene expression of corticotropin-releasing hormone, proopiomelanocortins, prolactin and somatolactin indicating lower stress pathways activation

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1060 Table 2. Studies investigating the effects of polymer-based water conditioners (adapted from Harnish *et al*. 2011).

Species	**Life stage/Size**	**Water conditioner**	**Concentration**	**Stressor**	**Effect**	**Reference**
Smallmouth bass (*Micropterus dolomieu*)	Adult	Catch'n'Release Formula®	5 g l^{-1}	Live release angling tournaments	Cardiac disturbances recovered within ~60min for control fish. Cardiac disturbances in fish exposed to Catch'n'Release lasted for ~180min	Cooke *et al*. (2002)
Delta smelt (*Hypomesus transpacificus*)	mean: 4.7 (August) 5.1 cm (November)	NovAqua® in 8 g l^{-1} NaCl	0.5 ml l^{-1}	Holding and transport post-capture	NovAqua in 8‰ NaCl increased 72 h survival (54.8%) when compared to the control with 8‰ NaCl (27.9%).	Swanson *et al*. (1996)
Largemouth bass (*Micropterus salmoides*)	Unreported	Unspecified commercial product§	1 mg 75 l^{-1} water	Live release angling tournaments	Survival for fish held in water with conditioner for 3-9 h was higher (96.5%) than fish held in unconditioned water (90.8%).	Plumb *et al*. (1988)

[§]The water conditioner contained unspecified quantitiesfosodium chloride, potassium chloride, sodium thiosulfate, pyrogenic silica, dimethylketone, alpha-methylquinoline, methylene blue, nitromersol, ethylenediaminetetraacetate, triethyleneglycol, and acriflavine (Plumb *et al*. 1988).

1064 Table 3. Research into the effects of adding NaCl to the water of fishes during transport.

Species	**Life stage/Size**	**Concentration**	**Stocking density**	**Stressor**	**Effect**	**Reference**
Brown trout (*Salmo trutta*)	mean: 76.2 ± 1.7 g, 20.4 ± 0.1 cm.	0.6 g l^{-1} NaCl	100 g l^{-1}	14 h transport	Smaller increase of blood oxygen carrying capacity than fish in the control group. No reduction of plasma osmolality and higher plasma levels of Cl^- and Na^+. Higher liver glycogen and muscle lipid contents when compared to control fish.	Nikinmaa *et al*. 1983

Freshwater drum (*Aplodinotus grunniens*)	mean: 36.5 cm	5 g l^{-1} NaCl	60 g l^{-1} 120 g l^{-1}	6 h transport	Reduction in immediate and delayed mortality after transport.	Johnson & Metcalf 1982
Striped bass (*Morone saxatilis*)	mean ± SE:72 ± 2.5 g	0 and 1 g l^{-1} NaCl	180 g l^{-1}	5 h transport	Reduction in delayed mortality over a 4-week period following transport. Lower plasma cortisol, glucose and potassium concentrations, and higher plasma sodium and chloride concentrations during recovery.	Mazik *et al*. 1991
White bass (*M. chrysops*) x striped bass hybrids	mean ± SE: 38.7 ± 1.1 g and 58.7 ± 1.1 g	Fresh water (5, 10, 20, 40, 80 mg l^{-1} Ca^{2+}) Salt water (1,	36.4 g l^{-1} 55.2 g l^{-1}	6.5 h confinement	Highest survival for fish in fresh water was at 80 mg l^{-1} Ca^{2+} at both stocking densities (with higher survival at higher	Weirich *et al.* 1992

	8, 16, 24 g l^{-1} NaCl)	stocking). Highest survival in salt water was at 8 g l^{-1} NaCl, with similar survival at both densities. Plasma osmolality decreased during confinement in fresh water. In sea water, fish in 8 g l^{-1} maintained plasma osmolality. Plasma osmolality at 16 and 24 g l^{-1} increased.

	mean group weights = 3.7 ± 0.3 to 55.8 ± 1.7 g	Fresh water (5, 10, 20, 40, 80 mg l^{-1} Ca^{2+}) Salt water (1, 8, 16, 24 g l^{-1} NaCl)	60 g l^{-1}	12 h simulated transport	Fish in all treatments had <5% mortality with no variation in mortality levels between the treatments.	
Xenocara (*Ancistrus triradiatus*)	mean: 10.4 ± 4.6 g	0.5, 1 g l^{-1} NaCl	61.75 g l^{-1}	48 h transport	Lower blood glucose levels after transport in both 0.5 and 1 g l^{-1} NaCl groups compared to levels in fish transported in fresh water or with zeolites. No significant difference between 0.5 and 1 g l^{-1} NaCl	Ramírez-Duarte *et al.* 2011

					groups. Lower mortality immediately post transport and 7 days post transport in both 0.5 and 1 g l^{-1} NaCl groups compared to levels in fresh water or with zeolites. No significant difference between 0.5 and 1 g l^{-1} NaCl.	
Xenocara (*Ancistrus triradiatus*)	mean: 9.0 ± 6.4 g and 7.0 ± 1.4 cm	1 or 2 g l^{-1} NaCl	137.5 g l^{-1}	12 h simulated transport	Lower blood glucose concentrations. Reduction in mortality following transport.	Ramírez-Duarte *et al.* 2013

		1 or 2 g l^{-1} NaCl	82.3 g l^{-1}	48 h simulated transport	Lower blood glucose concentrations but higher mortality during the 7 day post transport recovery period in the 2 g l^{-1} NaCl treatment.	
Astyanax altiparanae	Fingerlings (mean: 0.37 ± 0.05 g)	0, 3, 6, 9, 12, and 15 g l^{-1} NaCl	0.37 g ± 0.05 l^{-1}	96 h salinity exposure with no food.	All fish survived for up to 6 h in 0, 3, 6, and 9 g L^{-1} NaCl. After 96h, morality was 75% on the 9 g L^{-1} NaCl and 100% in the 12, and 15 g L^{-1} NaCl.	Salaro *et al*. 2015
		0, 3, 6, and 9 g l^{-1} NaCl	22, 30 and 37 g l^{-1}	8 h simulated transport	Blood glucose levels were significantly lower in the 30 and 37 g l^{-1} fish transported in the 3, 6, 9 g l^{-1} NaCl compared to the 0 g l^{-1} NaCl.	

Rainbow trout (*Oncorhynchus mykiss*)	mean: 200 g	0.5 g l^{-1} NaCl	Unknown	5 h transport	No increase in plasma glucose levels, compared to fish transported in fresh water where transport elevated plasma glucose. Fish transported in 0.5 NaCl g l^{-1} had a thinner mucus layer than fish transported in fresh water (see **Water Conditioners**)	Tacchi *et al*. 2015

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