Plasma exosomes are enriched in Hsp70 and modulated by stress and cortisol in rainbow trout

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Abstract
Exosomes are endosomally derived vesicles that are secreted from cells and contain a suite of molecules, including proteins and nucleic acids. Recent studies suggest the possibility that exosomes in circulation may be affecting recipient target cell function, but the modes of action are unclear. Here, we tested the hypothesis that exosomes are in circulation in fish plasma and that these vesicles are enriched with heat shock protein 70 (Hsp70). Exosomes were isolated from rainbow trout (Oncorhynchus mykiss) plasma using differential centrifugation, and their presence was confirmed by transmission electron microscopy and the exosomal marker acetylcholinesterase. Plasma exosomes were enriched with Hsp70, and this stress protein was transiently elevated in trout plasma in response to a heat shock in vivo. Using trout hepatocytes in primary culture, we tested whether stress levels of cortisol, the principle corticosteroid in teleosts, regulates exosomal Hsp70 content. As expected, a 1-h heat shock (+15°C above ambient) increased Hsp70 expression in hepatocytes, and this led to higher Hsp70 enrichment in exosomes over a 24-h period. However, cortisol treatment significantly reduced the expression of Hsp70 in exosomes released from either unstressed or heat-shocked hepatocytes. This cortisol-mediated suppression was not specific to Hsp70 as beta-actin expression was also reduced in exosomes released from hepatocytes treated with the steroid. Our results suggest that circulating Hsp70 is released from target tissues via exosomes, and their release is modulated by stress and cortisol. Overall, we propose a novel role for extracellular vesicular transport of Hsp70 in the organismal stress response.

Introduction
Recent studies have established exosomes, membrane-bound vesicles of endosomal origin, as important mediators of intercellular signaling (reviewed by Colombo et al. 2014). These extracellular vesicles are approximately 30–100nm in diameter and contain protein and nucleic acids, reflective of their cell of origin. Despite differences in cell types, exosomes have several common characteristics, including enrichment of sphingomyelin and tetraspanins (Rana & Zoller 2013, Andreu & Yáñez-Mó 2014) and heat shock proteins (Hsps), notably Hsp70 and Hsp90 (Thery et al. 2001, Clayton et al. 2005, Lancaster & Febbraio 2005, McCready et al. 2010). Recent studies suggest that...
exosomes from one cell type may communicate with a different recipient cell (Mittelbrunn et al. 2011). Most of these studies have utilized mammalian cell systems, and there is a paucity of information on exosome dynamics in whole organisms, as well as in lower vertebrates. A recent study, using a proteomics approach, reported Hsp70 and Hsp90 in exosomes in conditioned media derived from Atlantic salmon (Salmo salar) head kidney leukocytes (Iliev et al. 2010).

Heat shock proteins are a family of highly conserved proteins and play a key role in protein homeostasis during cellular stress (Deane & Woo 2011). In fish, the intracellular induction of Hsps, in particular Hsp70, has been well characterized in response to a plethora of stimuli (Iwama et al. 1999, Basu et al. 2002, Vijayan et al. 2010, Deane & Woo 2011). Also, studies have shown that cortisol, the primary circulating corticosteroid in teleost, modulates target tissue Hsp70 expression in fishes, thereby linking the cellular stress response with the organismal stress response (Ackerman et al. 2000, Basu et al. 2001, Boone & Vijayan 2002, De Boeck et al. 2003). Recent studies suggest that Hsp70 may also be released extracellularly in fishes, but the secretory pathways are far from clear (Iliev et al. 2010, Zhang et al. 2011). Hsp70 lacks the leader sequence necessary to be classically exported from the cell (Mambula et al. 2007), leading to the proposal that they are exported out of the cell in exosomes (Hunter-Lavin et al. 2004, Lancaster & Febbraio 2005). Although this has been confirmed in exosomes derived from human blood plasma (Kalra et al. 2013, Cheng et al. 2014, Vicencio et al. 2015) and mammalian cell culture media (Lancaster & Febbraio 2005, Merendino et al. 2010, Takeuchi et al. 2015), such studies are lacking in lower vertebrates (Iliev et al. 2010).

A recent study revealed that exosomal Hsp70 transmission across cell types may be a key mechanism for the maintenance of organismal proteostasis in Drosophila melanogaster (Takeuchi et al. 2015). To this end, Hsp70-positive exosomes have been shown to be cardioprotective by initiating intracellular secondary signaling cascades involving toll-like receptor 4 (Vicencio et al. 2015). To the best of our knowledge, this has yet to be shown in vivo in vertebrates. Given that neither circulating exosomes nor plasma Hsp70 have been reported in fish, we tested the hypothesis that circulating Hsp70 in fish are enriched in exosomes using rainbow trout (Oncorhynchus mykiss) as a model. To date, no study has addressed whether corticosteroids affect exosome release and/or packaging in any organism. As corticosteroid is a key player in the organismal stress response, we also tested the possibility that this hormone may modulate exosomal Hsp70 expression using trout hepatocytes in primary culture (Faught & Vijayan 2016).

**Materials and methods**

**Experimental fish**

*In vivo* heat shock study was carried out with juvenile rainbow trout (110 ± 20 g body mass) obtained from Alma Research Station (Alma, Ontario, Canada) and maintained at the University of Waterloo Aquatic Facility (Waterloo, Ontario, Canada). The fish used for exosome experiments (plasma exosomes and hepatocyte exosomes) were juvenile rainbow trout (150 ± 25 g body mass) obtained from Allison Creek Brood Trout Hatchery Station (Crownest Pass, Canada) and maintained at the University of Calgary Animal Care Facility (Calgary, Alberta). Fish were maintained at 12 ± 1°C on a 12-h light: 12-h darkness cycle and fed once daily to satiety with commercial trout pellet (Martin Mill, Elmira, Canada). The fish were acclimated for 2 weeks before the experiments. All experiments were approved by the Animal Care and Use Committee at the University of Waterloo and the University of Calgary and were in accordance with guidelines established by the Canadian Council for Animal Care.

**Whole animal study**

To assess the circulating Hsp70 levels in response to an acute heat shock, groups of 12 fish each were separated into four 100L tanks in a static system. Water in two tanks was heated to a temperature of approximately 25°C (+12°C above ambient), whereas that in other two tanks was maintained at ambient temperature (13°C). Fish were subjected to the +12°C heat shock for one hour after which they were brought back to 13°C within 15 min. Six fish from each of the heat-shocked and control (no heat shock) groups were sampled immediately after heat shock (1 h) and at 4 h and 24 h after heat shock. Fish were quickly netted and killed with an overdose of 2-phenoxyethanol (1:1000 well water; Sigma). Pieces of liver and blood samples were collected at each time point. Blood was collected in tubes containing EDTA (5 mM in Hank’s medium) and centrifuged for five min at 3000 g to separate the plasma. All samples were stored at −80°C for analysis later.
Hepatocyte study

Trout hepatocytes were isolated as described previously using in situ collagenase perfusion (Boone & Vijayan 2002). Hepatocyte viability was >95%. Cells were suspended in L15 (Gibco, Life Technology) medium and plated in six-well tissue culture plates (Sarstedt, Inc., Newton, MA, USA) at a density of 1.5 million cells/well (0.75 million cells/mL). Cells were allowed to recover for at least an hour, and then treated with either a vehicle (0.01% ethanol) or cortisol (100 and 1000 ng/mL). Treated cells were maintained at 11°C for 24 h at which time the L15 media was replaced and cells were again treated as previously. The cells either remained at 11°C or were subjected to a 1 h heat shock (+15°C above ambient) and allowed to recover for 24 h. This experimental protocol was previously used in Boone & Vijayan (2002) to assess the effect of cortisol on Hsp70 expression in trout hepatocytes. Cells were collected and media was removed and fractionated as described below to obtain exosomes. Experiments were repeated with hepatocytes from five independent fish.

Exosome fractionation

Plasma exosomes were isolated by differential centrifugation as described previously for fish cells (Iliev et al. 2010) and mammalian cell supernatants (Thery et al. 2006). Briefly, blood from each fish was collected in tubes containing a final concentration of 5 mM EDTA and centrifuged at 500 g to remove red blood cells and peripheral blood leukocytes (Fig. 1A). An aliquot of the plasma was sampled (total plasma t=0), and the remaining plasma was then subjected to two sequential centrifugation steps to remove cell debris: 1200 g for 20 min and 10,000 g for 30 min. This fractionated plasma was further spun at 150,000 g using an ultracentrifuge (Beckman) for 120 min to pellet the exosomes (Fig. 1A). The exosome pellet was resuspended in 1× PBS (20 µL/1 mL of plasma or 10 µL/1 mL of media fractionated). The supernatants and the exosome pellets were stored separately at −80°C for later analysis. The fractionation of exosomes from supernatants of hepatocyte cultures was also carried out exactly as described previously.

Transmission electron microscopy (TEM)

Freshly isolated exosomes in PBS were transferred to carbon-coated formvar films on copper-grids for 5 min, washed in distilled water for 20 s and treated with 1.8% methylcellulose/0.3% uranyl acetate for 4 min before drying. The samples were examined with a Tecnai F20 Transmission Electron Microscope (FEI, Hillsboro, OR, USA).

Hsp70 immunoblotting

Protein quantification, SDS-PAGE and western blotting were performed as described previously (Boone & Vijayan 2002). Total plasma protein concentration was determined by the bicinchoninic acid (BCA) method using

Figure 1

Exosome identification in plasma. Plasma from rainbow trout was fractionated by differential centrifugation to confirm the presence of exosomes (A). Exosomes were confirmed by TEM from freshly isolated plasma (B; arrows indicate exosomes; the scale bar denotes 100 nm). The average size as measured over 50 vesicles was 53.8 nm (±3.5 nm). Acetylcholinesterase (AChE) enzyme enrichment of the plasma exosomes (C; n=6 independent fish) was confirmed by comparing their presence in the plasma and the supernatant; bars with different letters are significantly different (P<0.05, one-way RM ANOVA).
bovine serum albumin standards. Plasma and hepatocyte homogenate samples were diluted to 1 mg/mL in a SDS sample buffer (0.06 M Tris–HCl (pH 6.8), 20% (v/v) glycerol, 0.02% (w/v) SDS, 0.025% (w/v) bromphenol blue and 5% B-mercaptopethanol). Samples were loaded on an 8% polyacrylamide gels with a molecular marker to confirm protein mass (Frogga Bio, Mississauga, Canada). Proteins were separated (200 V for 50 min; Mini Protean III (Bio-Rad) using a discontinuous buffer. The separated proteins were transferred to a 0.45 μm pore size nitrocellulose membrane (Bio-Rad) using a Transblot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) and transfer buffer (25 mM Tris (pH 8.3), 192 mM glycine, 20% v/v methanol). Equal loading and transfer efficiency was confirmed by Ponceau S. Blots were probed for 1 h at RT with anti-salmon Hsp70 (Boone & Vijayan 2002; StressMarq (SPC-313), Victoria, Canada) diluted to 1:1000, followed by anti-rabbit IgG-conjugated horseradish peroxidase (HRP) (1:3000; Bio-Rad). The protein band was detected using ECL Plus Western blotting detection reagent (Bio-Rad) and scanned using the Syngene G-Box Imager (Syngene, Fredrick, USA). Protein band intensity was quantified using ImageJ. Densometric values for plasma and exosome fractions were normalized to total lane protein detected with Ponceau S. Equal loading of hepatocyte samples was confirmed by incubation of membranes with Cy3-conjugated monoclonal mouse beta-actin antibody (Sigma, 1:1000) for 1 h at RT (Dindia et al. 2013). There was no change in beta-actin expression among treatments (data not shown).

Hsp70 enzyme-linked immunosorbent assay (ELISA)

Measurement of plasma Hsp70 by ELISA was carried out using a competitive sandwich ELISA adapted from Specker & Anderson (1994). Chinook salmon Hsp70 recombinant protein (Enzo Life Sciences (ADI-SPP-763-D), Exeter, UK) was used to coat wells, to which was added previously incubated samples or standards (1:1 ratio of homogenate to polyclonal rabbit anti-salmon Hsp70 primary antibody (1:100,000); StressMarq (SPC-313)). After overnight incubation, wells were washed and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000; Bio-Rad) diluted in 5% skim milk was added to each well. After a 1-h incubation and washings, the detection reagent was added (41 mM TMB in 200 mM potassium citrate, pH 4). After 1 h, the reaction was stopped with 8.5 M acetic acid in 0.5 M sulfuric acid. Wells were read at 450 nm using a multi-well spectrophotometer (VersaMax, Molecular Devices, Sunnyvale, USA).

Plasma cortisol

Plasma cortisol levels were analyzed using a 3H-cortisol radioimmunoassay that was validated for rainbow trout plasma as described previously (Sandhu & Vijayan 2011).

Enzyme activity

To assess cell leakage due to heat shock, the activity of the intracellular enzymes in the plasma (LDH and AspAT) was measured in 50 mM imidazole-buffered enzyme reagent (pH 7.4) at room temperature (23.5–24.5°C) by continuous spectrophotometry at 340 nm using a microplate reader as described previously (Ings et al. 2011).

Acetylcholinesterase (AChE) activity was measured as a marker of exosome release (Johnstone et al. 1987). The AChE protocol was adapted from Johnstone and coworkers (Johnstone et al. 1987) and Multhoff (2007) and measured at 415 nm for 30 min at room temperature. The extinction coefficient used to calculate AChE activity was 13,600 M/cm (DNTB, Ellman 1958).

- Lactate dehydrogenase (LDH: EC 1.1.1.27): 0.12 mM NADH and reaction started with 25 mM sodium pyruvate.
- Aspartate aminotransferase (AspAT: EC 2.6.1.1): 7 mM α-ketoglutarate, 0.025 mM pyridoxal 5-phosphate, 0.12 mM NADH and 8 U/mL malate dehydrogenase; reaction started with 40 mM aspartic acid.
- Acetylcholinesterase (AChE: EC 3.1.1.7), 0.1 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DNTB/Ellman’s reagent). The reaction was started with 10 mM acetylthiocholine.

Statistical analysis

Statistical analysis was carried out with SigmaPlot (Systat Software Inc, San Jose, USA). Exosome characterization of AChE and Hsp70 enrichment utilized a repeated measures (RM) one-way ANOVA. Significant differences were compared using the Holm–Sidak post hoc test with the level of significance set at P<0.05. The in vivo experiment utilized a two-way ANOVA. The in vitro hepatocyte experiment utilized a RM two-way ANOVA. The data were transformed, wherever necessary, to meet the assumptions of homogeneity of variance, although non-transformed data are shown in the figures. Results are presented as mean± standard error of mean (s.e.m.) for all groups, and the sample size is indicated in the figure legends.
Results

Plasma exosome characterization

Exosomes were isolated from trout plasma by differential centrifugation as outlined in Fig. 1A. The exosomes were confirmed microscopically using TEM (Fig. 1B) and enzymatically using acetylcholinesterase (AChE) (Fig. 1C). Exosomes in trout plasma were on an average 54 ± 4 nm in diameter (n = 50 exosomes; Fig. 1B). AChE activity was significantly enriched in the exosomes compared to the supernatant and total plasma fractions (Fig. 1C; P < 0.001, RM one-way ANOVA). The supernatant fraction had significantly less AChE activity compared to the total plasma fraction (P < 0.05; Fig. 1C).

Hsp70 in plasma exosomes

Hsp70 was significantly enriched in the exosomes compared to the supernatant and plasma fractions (Fig. 2). There was a >6-fold enrichment of Hsp70 expression in the exosomes compared to total plasma (P < 0.005; RM one-way ANOVA) and the supernatant (P < 0.001) fractions (Fig. 2). There was no significant difference in Hsp70 expression between the total plasma and the supernatant fractions.

Hepatocyte exosome release

Intracellular hepatocyte Hsp70 levels increased 7-fold after heat shock compared to controls (Fig. 3A; two-way ANOVA; F_{1,16} = 45.68; P < 0.001). Cortisol treatment had no significant effect (F_{2,16} = 0.17; P < 0.8) on hepatocyte Hsp70 expression (Fig. 3A). There was no significant interaction between cortisol treatment and heat shock (F_{2,16} = 0.012; P = 0.99).

Exosome Hsp70 expression was also significantly elevated after heat shock by 20-fold compared to controls (Fig. 3B; two-way ANOVA; F_{1,16} = 29.2; P < 0.001). Cortisol treatment significantly reduced the amount of Hsp70-positive exosomes (F_{2,16} = 4.73; P = 0.024). There was no significant interaction between heat shock and cortisol treatment (F_{2,16} = 0.32; P = 0.7). Multiple comparison analysis (Holm–Sidak method) revealed that 1000 ng/mL of cortisol significantly reduced Hsp70-positive exosomes (P = 0.023), whereas there was no significant difference between control and the 100 ng/mL cortisol group (P < 0.2).

There was no effect of heat shock on beta-actin expression in exosomes (Fig. 3C; two-way ANOVA; F_{1,16} = 1.61; P = 0.22). Cortisol treatment significantly reduced beta-actin expression in exosomes (two-way ANOVA; F_{2,16} = 17.0; P < 0.001). There was no significant interaction between heat shock and cortisol treatment on exosomal beta-actin expression (two-way ANOVA; F_{2,16} = 0.16; P < 0.9). Treatment with cortisol at both 1000 ng/mL (P = 0.002) and 100 ng/mL (P = 0.013) significantly reduced beta-actin expression in the exosomes compared to the controls (pairwise multiple comparison; Holm–Sidak method). Non-heat-shocked cells released exosomes with significantly lower beta-actin expression at both 1000 ng/mL (P = 0.012) and 100 ng/mL (P = 0.013) (pairwise multiple comparison) cortisol groups. Similarly, heat-shocked cells also released exosomes that had significantly reduced beta-actin expression in the 1000 ng/mL (P = 0.002) and 100 ng/mL (P = 0.007) cortisol groups compared to the control group (pairwise multiple comparison).

Whole animal study

Plasma cortisol levels were significantly higher in the heat shock group compared to the control group (Table 1;
Hepatocyte exosome release. Primary culture of hepatocytes were exposed to a 1 h heat shock (+15°C above ambient) and allowed to recover for 24 h at ambient temperature (11°C). Cells were treated with either a vehicle control (0.01% ethanol) or stressed level of cortisol (100 ng/mL and 1000 ng/mL). Intracellular Hsp70 expression was confirmed by immunodetection (A; n=5 independent fish hepatocytes; polyclonal rabbit anti-salmonid Hsp70: 1:1000; t-test). A representative western blot of Hsp70 and beta-actin (42 kDa) expression in these samples is shown below the histogram. Media from these cultured hepatocytes was fractionated and the expression of Hsp70 (B) and beta-actin (C) in exosomes confirmed by immunodetection (n=3–4 independent fish hepatocytes); bars with different letters are significantly different (P<0.05, two-way ANOVA). Treatment effects are shown as an inset in each figure. A representative western blot of Hsp70 and beta-actin (42 kDa) expression in these samples is shown below their respective histograms.

Table 1  Plasma protein, enzymes and cortisol levels. Plasma was sampled from trout that were either unexposed (control) or exposed to a 1 h heat shock (+12°C above ambient temperature) and then allowed to recover at ambient temperature (13°C). Samples were collected at 1, 4 and 24 h after heat shock.

<table>
<thead>
<tr>
<th>Time post-stress (h)</th>
<th>Treatment</th>
<th>Protein (mg/mL)</th>
<th>AsAT (U/g)</th>
<th>LDH (U/g)</th>
<th>Cortisol (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>23.34 ± 2.16</td>
<td>0.34 ± 0.03</td>
<td>1.95 ± 0.21</td>
<td>27.9 ± 5.4</td>
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<tr>
<td></td>
<td>Heat shock</td>
<td>20.82 ± 0.80</td>
<td>0.36 ± 0.02</td>
<td>2.17 ± 0.23</td>
<td>26.6 ± 3.1</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>19.79 ± 4.38</td>
<td>0.26 ± 0.03</td>
<td>2.34 ± 0.36</td>
<td>14.0 ± 2.5*</td>
</tr>
<tr>
<td></td>
<td>Heat shock</td>
<td>22.45 ± 0.23</td>
<td>0.33 ± 0.03</td>
<td>2.35 ± 0.19</td>
<td>29.5 ± 4.2*</td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>19.95 ± 2.02</td>
<td>0.26 ± 0.01</td>
<td>1.92 ± 0.21</td>
<td>17.0 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>Heat shock</td>
<td>23.81 ± 1.02</td>
<td>0.36 ± 0.04</td>
<td>2.11 ± 0.14</td>
<td>24.2 ± 4.2</td>
</tr>
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All measurements are mean ± s.e.m. (n=6).

*Denotes a significant difference between heat shock and control within the sampling time period; *denotes a significant difference between 4-h control fish and 1-h control fish (P<0.05, two-way ANOVA).

AsAT, aspartate aminotransferase; LDH, lactate dehydrogenase.
Discussion

Our results demonstrate for the first time the presence of exosomes in fish circulation, and these extracellular vesicles were enriched with Hsp70. Although the tissue sources of the circulating exosomes are unknown, our results for the first time, in any model organism, suggest a role for the stress hormone cortisol in modulating Hsp70 expression in exosomes derived from trout hepatocytes. The focus of Hsp70-enriched exosomes in mammalian models, including humans, has been primarily as a potential biomarker for cancer (Gobbo et al. 2016). However, a recent study proposed a physiological role for exosomal Hsp70 enrichment in the organismal cross-tissue protein homeostasis (Takeuchi et al. 2015). The presence of circulating Hsp70-positive exosomes in human (Kalra et al. 2013, Cheng et al. 2014, Vicencio et al. 2015) and fish plasma (this paper), as well as in non-vertebrate species (Takeuchi et al. 2015) underscores the export of Hsp70 via exosomes as a highly conserved response. This together with our finding that plasma Hsp70 levels are transiently modulated after an acute heat stressor in vivo in rainbow trout underscores a physiological role for this exosomal protein in the organismal stress response.

Trout plasma exosomes were confirmed by established markers (Savina et al. 2002, 2003), including vesicular size with TEM and enrichment of the membrane-bound enzyme AChE. Exosomes are approximately 50–100 nm in diameter (Colombo et al. 2014), and the presence of membrane-bound vesicles was confirmed in trout plasma (~50 nm) by TEM (Fig. 1B). The other routinely used marker is AChE, and this enzyme was first identified in exosomes released from reticulocytes (Johnstone et al. 1987). Although the exosome payload consists of a suite of proteins, including AChE, beta-actin and Hsp70 and may reflect the tissue of origin (Mathivanan & Simpson 2009, Mathivanan et al. 2010, Colombo et al. 2014), enrichment of AChE has been considered an excellent indicator of membrane-bound vesicles in circulation (Savina et al. 2002, 2003, Lancaster & Febbraio 2005, Merendino et al. 2010). This is because AChE-enriched exosomes are predominantly released from hematopoietic cells and may be abundant in circulation, given its important role in the immune response (Gastpar et al. 2005). Consequently, enrichment of AChE in trout plasma exosomes supports the presence of vesicles derived from hematopoietic cells (Fig. 1C), suggesting a role for these exosomes in the fish immune response.

A key finding was that Hsp70 was highly enriched in the exosome fraction of trout plasma (Fig. 2) and in supernatants from hepatocyte cultures (Fig. 3B). Hsp70 is expressed in most cells studied to date in fish (Deane & Woo 2011). Although Hsp70 enrichment in exosomes was reported in the conditioned media from Atlantic salmon head kidney leukocytes (Iliev et al. 2010), our study is the first to show the enrichment of Hsp70 in
exosomes in fish plasma in vivo, as well as its modulation by stressors. Although the source of this Hsp70-enriched exosomes is hard to predict, clearly leukocytes (Iliev et al. 2010) and hepatocytes (present study) are involved in the extracellular vesicular release in fish. Teleost hepatocytes have been well characterized for their heat shock response, as well as the modulation of Hsp70 in response to cortisol stimulation (Faught & Vijayan 2016). Using this model cell system, we demonstrate for the first time the release of Hsp70-enriched exosomes as part of the heat shock response in trout hepatocytes (Fig. 3B). Cortisol reduced this exosomal Hsp70 content suggesting a role for this stress hormone in the modulation of extracellular Hsp70 protein levels during stress. As acute heat shock elevates plasma cortisol levels at the organismal level (Basu et al. 2001, Table 1), we propose that cortisol may be affecting the release of exosomes from target tissues, and this may be the reason for the reduced Hsp70 expression, independent of heat shock, in exosomes released from hepatocytes. This was further supported by the observation that exosomal beta-actin expression was also reduced in a similar fashion to Hsp70 with cortisol treatment (Fig. 3C). Clearly studies are warranted to quantify exosomal release during stress in fish, and in the current study, this was limited by the availability of a robust marker, unlike in mammalian systems (Colombo et al. 2014), for exosomal sorting by flow cytometry. Based on our results, either Hsp70 or beta-actin may be a suitable candidate for exosomal sorting, and this remains to be verified.

Using plasma Hsp70 level as a proxy for exosome abundance, we show for the first time a transient elevation in plasma Hsp70 content in response to an acute heat shock. Although the presence of Hsp70 in extracellular fluids, including blood is well established (see reviews by Calderwood et al. 2007, De Maio 2011), this study supports the modulation of this protein in vivo by an acute heat stressor in fish. Hsp70 in plasma exosomes of rats were reported to be associated with the exosomal membranes and act as a signaling molecule in cardiomyocytes (Vicencio et al. 2015). Specifically, exosomal Hsp70 provided cardioprotection by increasing phosphorylation of protein kinases (ERK 1/2 and Akt), suggesting a regulatory role of exosomes during cardiac stress (Vicencio et al. 2015, reviewed by Ibrahim & Marbán 2016). Additionally, it was reported that exosomal Hsp70 was necessary to reduce protein aggregation characteristic of polyglutamate disease in vitro (Neuro 2A cells) and in vivo (Drosophila; Takeuchi et al. 2015). Consequently, exosomal Hsp70 is thought to play an important role in cross-tissue stress protection and to maintain protein homeostasis (Takeuchi et al. 2015).

The extracellular increase in Hsp70 levels in response to heat shock in the present study was not due to cell lysis as there were no differences in the plasma levels of markers of cell lysis, including AspAT and LDH (Bury et al. 1997).

The temporal profile of the elevated plasma Hsp70 response resembles the liver expression of this protein after a heat shock suggesting increased secretion of Hsp70-enriched exosomes. However, plasma Hsp70 levels approach basal levels by 24h despite the tissue Hsp70 content remaining elevated at 24h after heat shock (Fig. 4A and B). This suggests regulation of tissue Hsp70 content release or increased turnover of this protein after a stress in fish. As the plasma cortisol levels were higher in the heat-shocked group only at 4 and 24h compared to the controls (Table 1), we propose that this steroid may be playing a role in the transient modulation of plasma Hsp70 content after stress. Specifically, the observation that cortisol reduces Hsp70-enriched exosomes from hepatocytes in vitro (Fig. 3B), leads us to propose a role for this steroid in reducing plasma Hsp70 content after a heat shock, and this may include a reduction in exosomal release from target tissues. The physiological implications associated with exosome regulation by cortisol in response to stress remains to be determined. Although the mechanism of cortisol action on vesicular sorting is unknown, this steroid modulates liver plasma membrane fluidity in trout (Dindia et al. 2012). Target cell membrane properties are thought to be involved in exosomal release because GW4869, an inhibitor of neutral sphingomyelinases (nSMase) in mammals, inhibits exosome release (Takeuchi et al. 2015). Inhibition of nSMase prevents the catabolism of sphingomyelin to ceramide, a lipid signaling intermediate known to decrease membrane fluidity (Krönke 1999) and essential for endosomal sorting (Trajkovic et al. 2008). Indeed, the release of exosomes from mammalian hepatocyte is modulated by stressors, including ethanol, a membrane fluidizer (Momen-Heravi et al. 2015, Nojima et al. 2016).

In conclusion, we identified exosomes in trout plasma in vivo and in media from hepatocytes in vitro. These exosomes were enriched in the stress protein Hsp70, and this protein was subsequently used as a marker of vesicular release in response to an acute heat shock in rainbow trout in vivo. Acute stress and the associated increase in the stress hormone cortisol, has the potential to modulate exosome abundance and/or its Hsp70 content. To the
best of our knowledge, cortisol modulation of exosomes has not been reported in any other organism. Overall, Hsp70-positive exosomes may be playing an important role as intercellular communication signal during stress. Taken together, this study underscores an important, but yet uncharacterized, role for exosome signaling in the organismal stress response.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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