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Stress in African catfish (*Clarias gariepinus*) following overland transportation

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Abstract Of the many stressors in aquaculture, transportation of fish has remained poorly studied. The objective of this study was therefore to assess the effects of a (simulated) commercial transportation on stress physiology of market-size African catfish (*Clarias gariepinus*). Catfish weighing approximately 1.25 kg were returned to the farm after 3 h of truck-transportation, and stress-related parameters were measured for up to 72 h following return. Recovery from transportation was assessed through blood samples measuring plasma cortisol, glucose and non-esterified fatty acids (NEFA) and gill histology. Also, the number of skin lesions was compared before and after transport. Pre-transport handling and sorting elevated plasma cortisol levels compared to unhandled animals (before fasting). Plasma cortisol levels were further increased due to transportation. In control fish, plasma cortisol levels returned to baseline values within 6 h, whereas it took 48 h to reach baseline values in transported catfish. Plasma glucose and NEFA levels remained stable and were similar across all groups. Transported catfish did not, on average, have more skin lesions than the handling group, but the number of skin lesions had increased compared to unhandled animals. The macroscopic condition of the gills was similar in control, transported and unhandled catfish; however, light microscopy and immunohistochemistry revealed atypical morphology and chloride cell migration normally associated with adverse water conditions. From our data, we conclude that transportation may be considered a strong stressor to catfish that may add to other stressors and thus inflict upon the welfare of the fish.

Keywords African catfish · Stress · Transport · Welfare · Cortisol · Allostasis

Wageningen Aquaculture is a consortium of IMARES (Institute for Marine Resources & Ecosystem Studies) and AFI (Aquaculture and Fisheries Group, Wageningen University), both part of Wageningen University & Research Centre (WUR).

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Introduction

The world's human population is increasing daily and so is the demand for food. A growing source of protein is fish. The amount of fish caught in the wild has become stable over the past years; the farming of

aquatic animals has been growing rapidly and is expected to continue to grow at least till 2025 (Diana 2009). The rapid growth of aquaculture activities causes public concern about the sustainability of the aquaculture sector.

An important aspect of sustainability is animal welfare. Although animal welfare has been defined in different ways (Spruijt et al. 2001; Korte et al. 2007; Broom 2011; Hagen et al. 2011; Ohl and van der Staay 2012), key to all definitions is that poor welfare is associated with overtaxing the adaptive capacity of animals (allostatic overload; McEwen and Wingfield 2003) which may result in chronic stress-related physiology and behaviour, pathology, and increased mortality. Successive or cumulative exposure to stressors may compromise the adaptive capacity of an animal and lead to allostatic overload and poor welfare (Korte et al. 2007; Ohl and van der Staay 2012). It is therefore important to identify the effects of stressors in aquaculture conditions. Globally, the production of African catfish is increasing rapidly, with an estimated global production of 194,000 tonnes in 2011 (http://www.fao.org/fishery/culturedspecies/Clarias_gariepinus/en). Here we studied the effects of (a simulated) transportation of an important species for Dutch aquaculture, namely the African catfish (*Clarias gariepinus),* from farm to slaughterhouse, as (in general) transport is still poorly understood in terms of stress (Dalla Villa et al. 2009).

During transport, fish are exposed to a multitude of stressors such as density changes, handling stress, water movement, noise, vibrations and poor water conditions. Exposure to such stressors simultaneously or in rapid succession may induce severe physiological stress (McEwen and Wingfield 2003; Koolhaas et al. 2011). For land animals, it has been clearly shown that transportation under inappropriate conditions causes severe stress and leads to poor welfare (Warriss 1998; Broom 2005). The limited number of studies in fish species indicates that transport increases plasma cortisol levels in Atlantic salmon (Iversen et al. 2005), Rainbow trout (Chandroo et al. 2005) and Stingray (Brinn et al. 2012; Nikoo and Falahatkar 2012). While in these studies, growth rate and mortality were not affected following transport, it was not clear how levels of cortisol changed following transport. To add to this (limited) body of knowledge on stress in fish following transport, we simulated an overland transportation of the African catfish and studied stress physiology and recovery.

Market-size catfish were transported for 3 h in a commercial setting and returned to the farm (instead of ending at the slaughterhouse). Upon return, the following stress-related parameters were measured: plasma levels of cortisol, glucose (Martinez-Porchas et al. 2009) and non-esterified fatty acids (NEFA) (Rosen and Spiegelman 2006) as well as gill morphology (Roques et al. 2010) and number of skin lesions (Øverli et al. 2002; Summers and Winberg 2006; Craig 2007). One group was sampled directly upon return (T0), while four other groups were sampled at 6, 24, 48 and 72 h following transport. Controls were held under identical holding conditions, but they were not transported and remained at the farm.

Materials and methods

Ethics

All experiments were approved by the Animal Ethics Committee of Wageningen UR and were conducted in agreement with Dutch laws (Wet op de Dierproeven 1996) and European regulations (Directive 86/609/EEC).

Weather conditions

The experiment was carried out in the Netherlands during spring (June 2010). Weather conditions at the day of transportation and during the week of sampling were approximately as follows: no rainfall, ambient temperature: 14 °C, clouded (5 octas) and an average wind speed of 3 m/s.

Experimental details

Of the 1,500 African catfish (*Clarias gariepinus*; mixed sexes; 1–1.5 kg) used in this study, 150 were killed for analysis; the remaining fish were sold for slaughter. Figure 1 shows a schematic overview of the experiment and experimental groups. A more detailed description is given hereafter.

The husbandry conditions at the farm for fish weighing 1–1.5 kg were stocking density up to 250 kg/m^3, water temperature of 27 °C and a 0:24-h light–dark cycle (continuous twilight); food was provided at 07.00, 15.00 and 23.00 h.

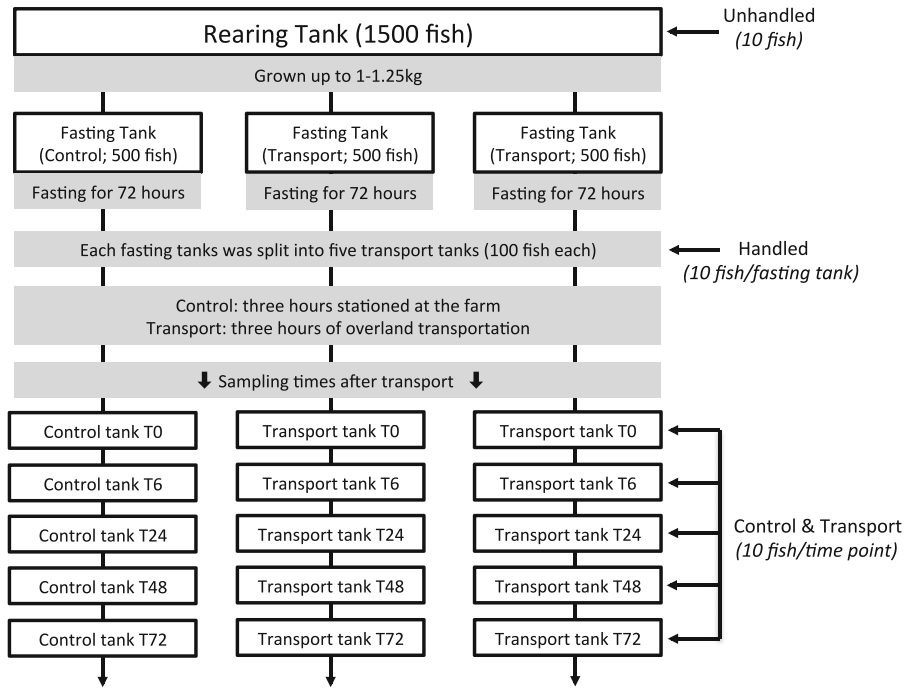


Fig. 1 Experimental set-up. Fish were sampled during standard housing 1 week prior to the experiment to assess unhandled plasma values, skin lesions and gill morphology. Directly after subdividing into transportation tanks, fish were sampled to assess stress physiology as a result of handling and fasting. After 3 h of transportation and return to the farm (transported fish), or stay at the farm (control fish), fish were sampled after 0, 6, 24, 48 and 72 h of recovery

The farmer who participated in this study focuses on the on-growing phase, which ranges from 10 to 1,500 g. The fish are reared in large concrete tanks, which are part of a recirculating aquaculture system (RAS). Depending on their life-stage, fish are kept in 4,000 or 12,000 l tanks. African catfish is an air-breathing fish, and therefore, the farmer does not monitor the oxygen levels in the tanks.

At the farm, the market-size catfish are fasted prior to transportation to the slaughterhouse. Food is being withheld for 24 h in the RAS; subsequently, animals are transferred to flow-through tanks (21–23 °C, pH 6.9, a 0:24-h light–dark cycle) where the fish are fasted for 48 h to remove undesirable off-flavours. After this 72-h fasting period, the farmer considers the fish to be ready for transport.

At the day of transport, the fish experienced a drop of approximately 40 cm when transferred from the, by now, drained flow-through tank to an empty container to weigh the fish before transport (subgroup or tank); the control group that remained at the farm was handled the same way.

Subsequently, each subgroup (125 kg fish) experienced a drop of approximately one metre during transfer to a partially filled (125 l) 600 l plastic tank (120 × 100 × 79 cm, grey pallet box; Hulkenberg,'s Heerenberg, the Netherlands). These tanks were similar in size and volume as the transportation tanks normally used by the slaughterhouse. In all groups (controls and transported), the density increased from 250 kg/m^3 (husbandry and fasting) to 500 kg/m^3 (transportation tanks). A stocking density of 500 kg/m^3 is a common practice during transportation of African catfish to a slaughterhouse in the Netherlands. Tanks were covered with a plastic export pellet (120 × 100 cm—nestable; Hulkenberg,'s Heerenberg, The Netherlands) to avoid escape and loaded onto the truck via a forklift. During transportation, tanks were not provided with aeration or temperature control. However, the truck was well insulated leading to limited loss of temperature (as shown in Table 1; directly after transport at T0).

Upon return, the truck was unloaded and each tank was supplied with fresh ground water (flowrate; 10 l/h) to lower density from 500 to 250 kg/m^3 as practiced and recommended by the farmer. For water outflow, each tank was equipped with an outlet; water was not recirculated, as this is practice for non-fed fish

Table 1 Water quality of water used at the farm and in our experiment

	Temp (°C)		O_2 (mg/l)		TAN (mg/l)		NO_2 (mg/l)		NO_3 (mg/l)		pH	
Ground water	21.5		2.0		2.14		0.003		0.03		7.3	
Fasting tanks	22.0		4.0		16.05		0.021		7		6.9	
	Control	Transport	Control	Transport	Control	Transport	Control	Transport	Control	Transport	Control	Transport
T0	21.5	20.0	6.0	6.0	13.10	11.76	0.05	0.22	5.0	3.1	7.6	7.2
T6	21.5	20.0	2.0	2.0	9.34	7.42	0.04	0.03	2.1	1.4	7.1	6.7
T24	21.5	21.5	3.0	3.0	9.93	8.87	0.02	0.02	1.0	0.8	7.4	7.4
T48	20.5	20.0	3.0	3.0	6.50	8.95	0.01	0.04	1.1	0.5	7.3	7.3
T72	21.0	20.0	3.0	3.0	7.01	2.72	0.04	0.06	1.6	0.7	7.3	7.3

Ground water was used as fresh water in both the fasting tanks and experimental tanks. Values shown are single measurements, taken moments before fish were taken out for sampling

in flow-through tanks at the farm. Control tanks were hooked up to the flow-through system after a 3-h wait.

Due to the limitations in space at the farm, only three groups (500 fish per group) could be included in the experiment. The authors have chosen for one control group and two transported groups.

Water quality

Samples to assess water quality were collected moments before fish were randomly taken from their holding tanks for sampling. Water quality was assessed on the basis of concentrations of total ammonia nitrogen (TAN, expressed as NH_3–N mg/l), nitrite (in NO_2^-–N mg/l) and nitrate (in NO_3^-–N mg/l). These parameters were analysed spectrophotometrically (Hach Lange Spectrophotometer DR 5000 UV/VIS; Hach Lange GmbH, Berlin, Germany), using protocols of Hach Lange. In addition, levels of dissolved oxygen (mg/l), pH and tank water temperature were analysed with a Hach Lange HQ40d multi-analyser (Hach Lange GmbH, Berlin, Germany).

Euthanasia and sample collection

At the time of sampling, ten fish were removed at random from the tanks by netting and placed within a water-filled tub containing 0.1 % (v/v) 2-phenoxyethanol (Sigma, St. Louis, MO, USA). Once deeply anaesthetized (always within 1 min), one ml blood was drawn with heparinized syringes and collected in reaction vials (Eppendorf, 1.5 ml) and immediately put on melting ice. Subsequently, reaction vials were centrifuged (14,000 rpm, 4 °C, 10 min), and blood plasma was separated from blood cells and stored at −20 °C until analysis.

Gill tissue was excised and put into 50 ml Greiner tubes containing BOUIN's fixative filtrated saturated picric acid, saturated formaldehyde (37 %), glacial acetic acid (15:5:1 ratio) and stored at room temperature over-night. The following day, the fixative was replaced with 50 % ethanol, which was refreshed once within 24 h.

After blood and gill tissue had been collected, the fish were killed by transection of the spinal cord just behind the skull.

Gill morphology and immunohistochemistry

Immunohistochemistry was done according to the methods of Metz et al. (2003). In brief, fixed tissue was dehydrated in serial ethanol concentrations and embedded in paraffin. Seven μm sections were mounted on gelatinized glass slides and dried. After paraffin removal, slides were placed in 2 % (v/v) H_2O_2 to neutralize endogenous peroxidase activity for use in immunohistochemistry. Subsequently, non-specific binding sites were blocked with 2 % (v/v) normal donkey serum, and the slides incubated overnight with monoclonal antibody against chicken Na+/K+-ATPase (IgGα5, Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, USA) at a final dilution of 1:300 (v/v). Goat anti-mouse (Nordic Immunology, Tilburg, The Netherlands) was used as secondary antibody at 1:200 (v/v) dilution. Slides were

subsequently incubated with the Vectastain ABC Kit (Vector Laboratories Inc., Burlingame CA, USA). Staining was performed in 0.025 % (w/v) 3,3′-diaminobenzidine (DAB) and 0.0005 % (v/v) H_2O_2.

The number of chloride cells was determined by averaging the number of cells counted [by two researchers (RM, JB)] on 20 lamellae of a single filament. A total of 6 filaments were analysed for each fish and ten individuals per group were used. For morphological comparison, we used previously published data from laboratory housed African catfish (Schram et al. 2010). Here, increasing levels of ammonia in the water caused deterioration of gill epithelia.

Skin lesions

At each of the sampling moments fish were checked for skin lesions. The number of skin lesions (including lesions on fins and barbless) was derived from two separate counts per fish performed by two researchers (RM, JB). Both new/recent (bloody) and old (scar tissue) lesions were included.

Plasma analysis

Cortisol was measured as previously described by Gorissen and colleagues (Gorissen et al. 2012). Briefly, 96-well microtiter plates were coated with mouse cortisol-antibodies in coating buffer. Plates were cleared of coating buffer and washed with a wash buffer before blocking possible non-specific binding sites with blocking buffer. Wells were cleared of blocking buffer and 10 µl of standard or sample, together with 90 µl of tracer, was added to the wells. After the incubation period wells were cleared and washed before scintillation liquid was added and radioactivity measured with a β-counter (detection limit: 4 ng/ml; inter-assay VC: 12.5 % and intra-assay VC: 2.5 %). Glucose and NEFA were measured using commercially available kits (Wako Chemicals USA Inc., Richmond VA, USA).

Statistics

Statistical analysis was performed using Kruskal–Wallis to test for significance over time. To test for significance between groups the Mann–Whitney *U*-test, unpaired *t* test or unpaired *t* test with Welch's correction was used, depending on sample distribution. Significance was set at $P \leq 0.05$ (two-tailed); all values are expressed as mean ± standard deviation (sd), unless otherwise indicated.

Results

Water quality

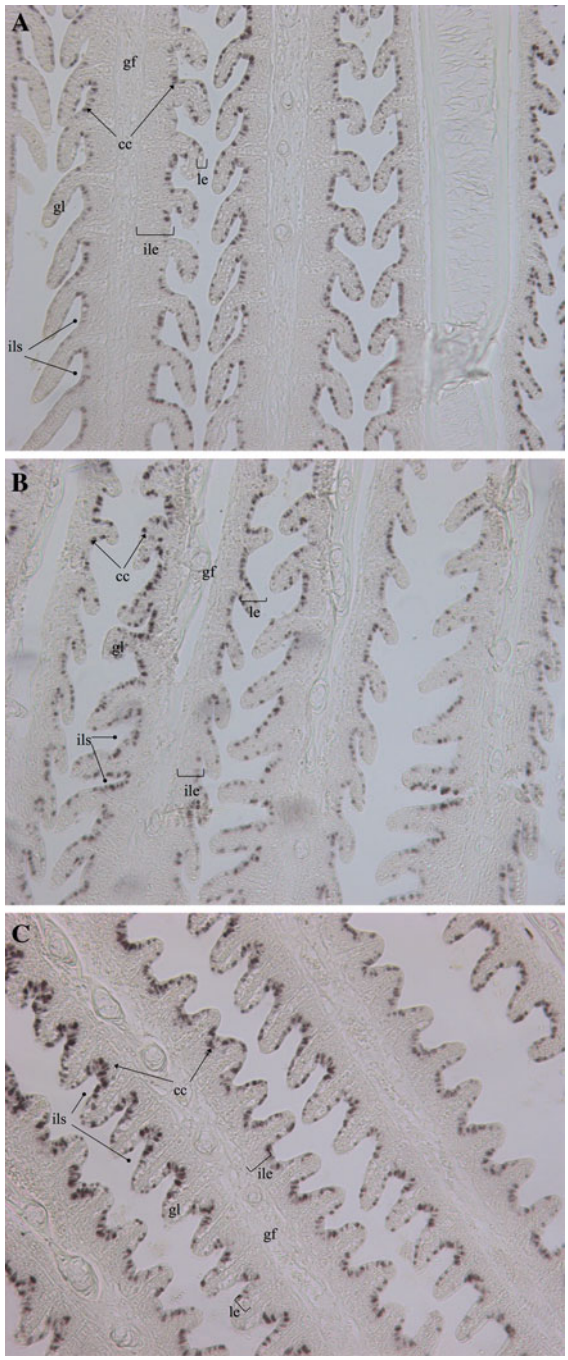
Throughout the experiment water quality was monitored (Table 1). During 3 h of transport, water conditions slightly deteriorated (compared to ground water), but remained within acceptable conditions (Belao et al. 2011; Hilmy et al. 1987; Schram et al. 2010, 2012). TAN, NO_2 and NO_3 were increased in both control and transport tanks after 3 h (T0). Although tanks were not provided with aeration of the water, oxygen levels increased during the first 3 h from 2.0 mg/l (ground water) to 6.0 mg/l (T0). Once tanks had been provided with fresh ground water (T6–T72), values returned to those found in the fasting tanks. The only exception to this is TAN, which remained lower (16.05 vs. 2.72–7.01 mg/l).

Gill morphology

Immunohistochemistry revealed that the position and number/density of chloride cells in the gills did not differ between transportation and control groups (Fig. 2a–c; data not shown). However, when compared to gill tissue taken from African catfish exposed to different levels of ammonia under laboratory conditions (Schram et al. 2010), gill morphology of these farmed fish appeared deteriorated in all groups (i.e. the condition of the gill epithelia was reminiscent of that of fish exposed to higher levels of waterborne ammonia). Compared to control animals from the ammonia experiment, farmed fish showed both thickened inter-lamellar and lamellar epithelium and a reduced inter-lamellar space. In addition, chloride cells were found to have migrated from the filament position towards the tips of the lamella.

Skin lesions

Transported fish did not have more skin lesions than those held under control conditions (Fig. 3). When



◀ **Fig. 2** Histology of gill epithelium immunohistochemically stained for Na^+/K^+-ATPase-rich cells (chloride cells) taken from **a** unhandled catfish, **b** directly after transportation (T0) and **c** 3 days after transportation (T72). No difference in morphology nor the number or location of the chloride cells was observed between groups (including those not shown, i.e. at T6, T24 and T48 and control groups). However, gill morphology in all fish looked somewhat deteriorated: thickened lamellar & interlamellar epithelium and chloride cells positioned outside the interlamellar epithelium (migration towards the tips of lamellae). *Legend*: *ile* interlamellar epithelium, *le* lamellar epithelium, *ils* interlamellar space, *cc* chloride cells, *gf* gill filament, *gl* gill lamella. ×400 magnification

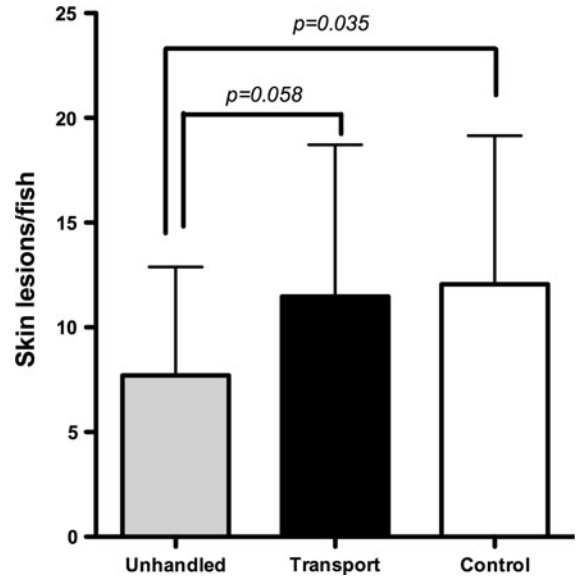


Fig. 3 The mean (±SD) number of skin lesions per fish for unhandled, control and transported fish. Lesions refer to both freshly sustained injuries and older (scar tissue) lesions. Unhandled: $n = 10$; control: $n = 50$; transported: $n = 100$

compared to conditions before transportation, however, the number of skin lesions (transported and controls: on average 12 lesions/fish) was 1.5-fold higher than prior to fasting (unhandled fish: on average 8 lesions/fish).

Stress parameters: cortisol

Before transportation plasma cortisol levels were low (unhandled; <10 ng/ml; Fig. 4). When fish were prepared for transport (handled), plasma cortisol levels significantly rose above unhandled levels (35 ng/ml). During 3 h of transport, plasma cortisol levels increased further to 50 ng/ml, while plasma cortisol levels in non-transported (controls) did not (30 ng/ml). Six hours later (T6), plasma cortisol levels in control fish had already returned to unhandled levels. In transported fish, however, cortisol levels in plasma remained significantly higher till 48 h after return to the farm. At that point values were also no

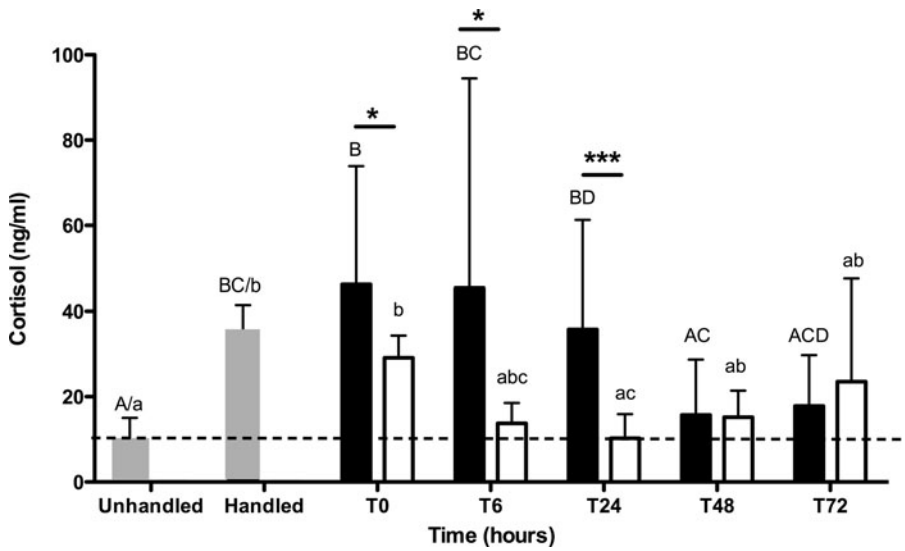


Fig. 4 Mean (±SD) plasma cortisol levels for catfish under standard conditions (unhandled; *grey*; *dotted line*), after fasting and handling (*grey*) and in control (*white*) and transported (*black*) groups during recovery. On-growing catfish have low levels of plasma cortisol (<10 ng/ml), which increase after sorting and readying for transportation (handling). After 3 h of staying at the farm plasma levels remained high in control fish, whereas plasma cortisol in transported fish increased even further (T0). During the 72-h recovery period, plasma levels return to baseline values at T6 (controls) and T48 (transported). Unhandled and control: $n = 10$; Handled: $n = 30$; transported: $n = 20$. $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) between transported and control at the same time point. Letters indicate significance over time within control (*lowercase letters*) or transported (*capital letters*) groups. Data-points with the *same letter* are not significantly different from each other

longer significantly different from the control group and unhandled group.

Stress parameters: glucose and NEFA

Plasma glucose (Fig. 5a) did not change significantly between controls and transported animals. After 48 h a significant drop in plasma glucose was observed in both the transported and control group compared to unhandled and handled animals. Similar results were obtained for plasma NEFA (Fig. 5b). NEFA did not differ significantly between the transported and control group, but over time plasma NEFA levels rose above unhandled levels in both the transported and control group (from T24 onwards in transported fish, from T6 onwards in controls).

Discussion

This experiment provides novel insights on the stress status of African catfish after a (simulated) commercial overland transport; experiments monitoring recovery after transport are rare and have been, to the best of our knowledge, only been performed in a few other fish species, i.e. Atlantic salmon (Iversen et al. 2005), Rainbow trout (Chandroo et al. 2005) and Stingray (Brinn et al. 2012; Nikoo and Falahatkar 2012). The data of our study show that (1) water quality remained within acceptable limits during a (simulated) commercial 3-h transport; (2) gill morphology was not altered as a result of transport, although gill morphology was deteriorated in all sampled fish; (3) the number of skin lesions had increased in transported and control fish compared to values in unhandled fish; (4) plasma cortisol levels were increased due to on-farm fasting followed by handling preceding transportation, and more strongly so, and for a longer period of time, when fish were transported in addition; (5) plasma glucose and NEFA levels remained unaffected by handling and transport, but were slightly changed after 48 h of recovery.

Even though transportation tanks were not accommodated with fresh water and fish density was high (500 kg/m^3 at transportation and 250 kg/m^3 at the farm), water quality only slightly deteriorated during the 3 h of transport (compared to ground water). The 72 h of fasting prior to transportation likely prevented

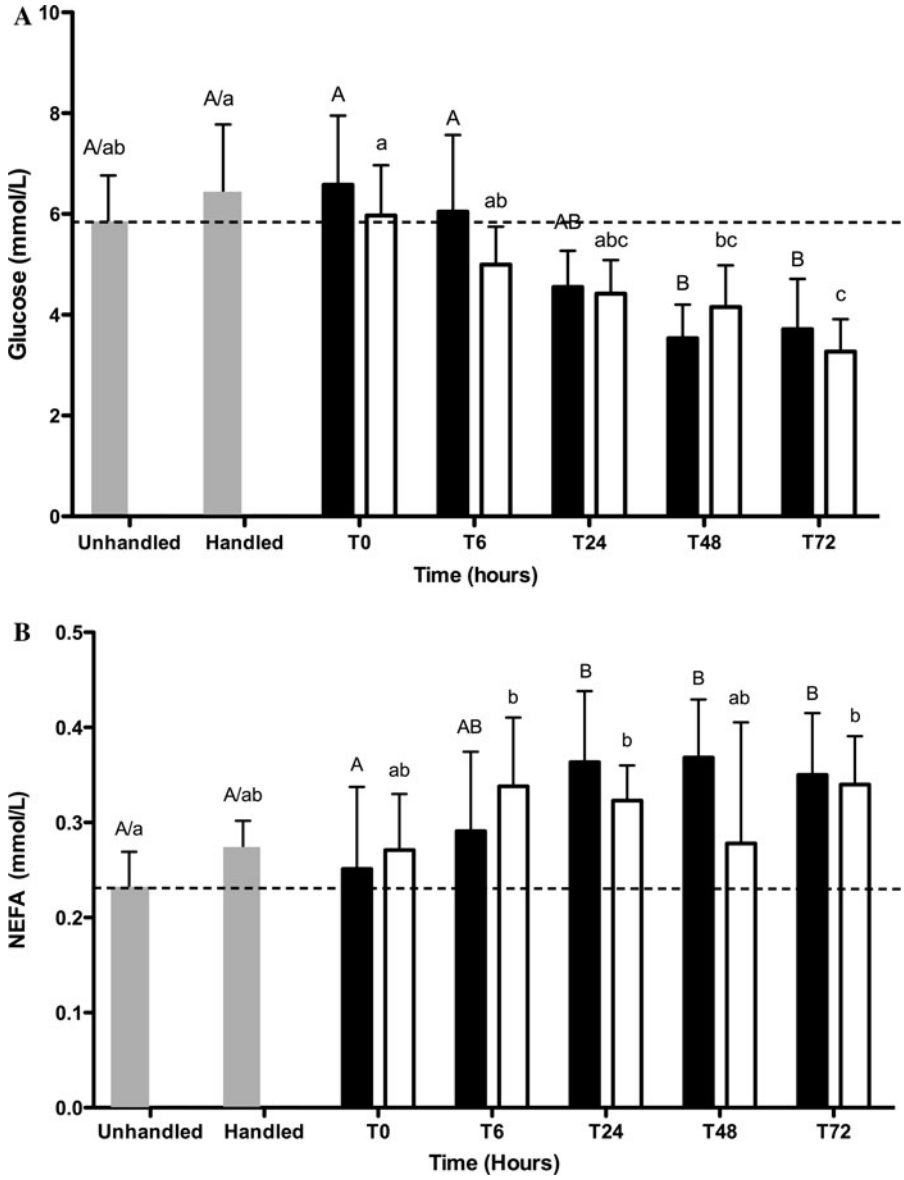


Fig. 5 Mean (±SD) plasma levels for **a** glucose and **b** NEFA for catfish under standard conditions (unhandled; *grey*; *dotted line*), after fasting and handling (*grey*) and in control (*white*) and transported (*black*) groups upon recovery Glucose and NEFA levels for on-growing catfish (basal) were 5.8 and 0.25 mmol/l, respectively. In both transported and control fish, plasma glucose remained stable, but dropped in both groups below unhandled conditions after 48 h. In contrast, plasma NEFA levels rose above unhandled values after 24 h of recovery (T24) in transported fish and 6 h (T6) in control fish. Unhandled and control: $n = 10$; Handled: $n = 30$; transported: $n = 20$. $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) between transported and control groups at the same time point. *Letters* indicate significance over time within control (*lowercase letters*) or transported (*capital letters*) groups. Data-points with the *same letter* are not significantly different from each other

a poor water quality. This fasting period ensured that fish did not have food in their digestive tract, which was indeed confirmed upon autopsy at sampling (data not shown). This prevented the fish from emptying their stomach when stressed, lowered their metabolism and, as a consequence, decreased the excretion of nitrogenous waste products by the fish to the water.

Calculated values of unionized ammonia (Emerson et al. 1975) did not exceed the criterion of 0.34 mg NH_3–N/l for this species (Schram et al. 2010). For African catfish nitrite-nitrogen levels should not exceed 1.5 mg/l in absence of chloride (Hilmy et al. 1987). Here, nitrite levels remained well below this value. Furthermore, although nitrate levels did increase 7-fold in both transportation and control tanks, they remained far below the recently proposed maximum of 140 mg/l nitrate-nitrogen (Schram, Roques et al. 2012). Temperature of the water in the tanks remained around a constant 20 degrees. Temperatures ranging between 18 and 28 °C occur in the natural habitat of this species (Bruton 1979). For dissolved oxygen no criterion for the African catfish has been published. It is known that for rearing at farms the levels of dissolved oxygen in water may vary between

0.5 and 3 mg/l (Desmares 1993). Here, all measured values for oxygen were substantially higher than the lower reported value of 0.5 mg/l. However, it has also been shown that an oxygen level of 3.2 mg/l (60 mmHg) or higher is desired for African catfish held at 25 °C. Higher concentrations do not significantly increase oxygen uptake from the water, whereas lower levels rapidly decreased VO_2 via the gills, which was compensated by increasing air-breathing frequency (Belao et al. 2011). Summarizing: values for unionized ammonia, pH, nitrate, nitrite and temperature were within the range that is considered not to affect the welfare of African catfish.

For fish it is known that, as a secondary response to stress, the morphology, incidence, frequency, density and the position of chloride cells in the gill epithelium may change compared to stress-free conditions (Harper and Wolf 2009; Roques et al. 2010). There was no difference in morphology, the number or the position of chloride cells between unhandled, controls and transported catfish. However, unexpectedly the overall gill morphology appeared somewhat deteriorated. When these gills were compared with gills from a previous experiment under laboratory conditions (Schram et al. 2010), they were reminiscent of those of fish exposed to elevated levels of unionized ammonia that exceeded the criterion of 0.34 mg NH_3–N/l. This suggests that the fish at the start of this experiment were reared under less than optimal water conditions preceding transportation. We did not measure TAN levels over 0.34 mg NH_3–N/l that could explain the deteriorated gill morphology. It is, however, possible that a prolonged intensive rearing and feeding history may have demanded morphological changes at the level of the gills related to feeding-associated spikes in nitrogenous waste. Although gill morphology was found to be deteriorated, fish were considered fit for travel as no signs of poor health were observed prior or during the experiment. Future experiments should determine whether deterioration of gill morphology affected the overall results of the experiment.

No differences in the number of skin lesions of transported fish compared to control fish were observed. However, when controls or transported fish were compared to unhandled fish (baseline), the number of skin lesions was increased. This could be due to injuries sustained during preparation for transportation, i.e. draining the holding tank of water and consequently increased density, the 40 cm drop into empty crates, weighing and exposing fish to a second drop of 1 metre into water-filled transportation tanks. Another cause could be aggressive acts between fish, either provoked by stress (Øverli et al. 2002; Summers and Winberg 2006; Craig 2007) or by the struggles to establish a new hierarchy (Pottinger and Pickering 1992), as groups were split into new tanks; disrupting existing and calling for a new hierarchy to be established.

Handling of the fish after the fasting period, but prior to transportation caused a mild, yet clear increase in plasma cortisol levels. Control animals did not show signs of recovery within the first 3 h after handling stress, as measured by the plasma cortisol levels directly after transport (T0). Previous experiments have shown that cortisol levels start to drop immediately after removing the stressor (a 50-min confinement) in channel catfish (Davis and Small 2006), but remain above baseline values for at least 100 min. Unpublished results from our laboratory have shown that cortisol levels returned to baseline values within 30 min after removing a stressor (15 min of air-exposure/crowding) in the African catfish.

A number of factors may have been responsible for the latency in recovery (9 h after handling) in the control group. At time point T6, plasma cortisol levels in control fish were no longer significant compared to unhandled fish. This is 6 h after fish density returned to a value of 250 kg/m^3 housing conditions (half the density of transport conditions). This could suggest that the change in density may have been a stressor in our set-up, causing increased levels of plasma cortisol. Secondly, fish were placed in a new environment when placed in the transportation tanks, which may have evoked anxiety and stress (Cachat et al. 2010) and it may have taken some time before fish were adapted to this new environment (Matsunaga and Watanabe 2010). Thirdly, the group composition was changed as fish were divided over (transportation) tanks and this could have instigated the formation of a new hierarchy. Higher levels of basal cortisol are known to be associated with hierarchy formation in mammals (Gust et al. 1991). This may be similar in fish. Fourthly, the stressor applied here may have been more severe (both in duration and intensity) than the stressor applied by Davis and Small (2006), leading thus to more elevated plasma cortisol levels. Fifthly, it is possible that plasma cortisol levels measured after 3 h were actually declining from a higher value earlier;

i.e. cortisol peak values could have been highest somewhere during the 3-h wait period at the farm. In the experimental setup, due to limitations in space at the farm, it was impossible to have included extra groups to control for these factors.

Compared to control fish, transported fish showed even higher levels of plasma cortisol directly after transport. In addition, it took longer (8-fold) for plasma cortisol levels to return to baseline values in transported fish. This (prolonged) recovery time was estimated as a 4 on a scale of 1–5 by Dalla Villa et al. (2009), where 1 is given as a mark to adverse effects lasting up to 3 h and 5 given as a mark for adverse effects more than 72 h. This indicates that overland transportation is, as anticipated, a stressful experience in line with data on transportation of land animals (Warriss 1998; Broom 2005). In contrast, cortisol levels during transportation of salmon in well-boats returned to normal during transportation (Iversen et al. 2005). Only when transportation took place during rough weather, which could be considered a stressor, did plasma cortisol levels remain elevated (Iversen et al. 2005). During overland transportation fish are exposed to additional stressors, such as vibrations coming from the truck, constant water movement and unpredictable (traffic) noise. Such additional stressors during overland transportation could explain the increase in plasma cortisol and prolonged recovery time observed in transported animals.

Unlike shown in previous studies (Martinez-Porchas et al. 2009), there was no significant increase in plasma glucose when fish were stressed (handled) compared to fish under baseline conditions. A possible reason could be that glucose levels had already decreased after the 72-h fasting period where food was withheld and temperature lowered and that the handling stress brought glucose back to unhandled levels. Unfortunately, due to the experimental design, i.e. (simulated) commercial practice, we were unable to take plasma samples directly after 72 h of fasting (prior to handling). During the course of the experiment, plasma glucose levels were steady in both transported and control fish, up till T48, where a decline was observed. At the same time, plasma cortisol levels slowly increased. It has been reported that cortisol levels rise when fish are fasted for longer periods of time (Mommsen et al. 1999)) and that this rise can be initiated by a drop in plasma glucose (Lado-Abeal et al. 2002). In addition it appeared that the plasma glucose levels were inadequate to provide all the energy needed, as there was a rise in plasma NEFA levels around the same time as the glucose levels dropped. Normally NEFA levels are low in healthy animals and an increase in NEFA levels is often associated with fasting and indicative of a negative energy balance (Rosen and Spiegelman 2006). Although it remains unclear whether our results result from fasting, it is not uncommon for plasma NEFA levels to increase during periods of fasting and this has been previously observed among others in common carp (*Cyprinus carpio*) (Huising et al. 2006).

Conclusions

Our observations provide novel insight in the status of the African catfish during a (simulated) commercial overland transport. The data show that overland transportation is a significant stressor requiring a recovery period of 48 h. Overland transportation exposes fish to a multitude of stressors that jointly may impose a significant allostatic load to the animals (Korte et al. 2007; Koolhaas et al. 2011; Ohl and van der Staay 2012). Cumulative effects of stressors could lead to allostatic overload, eventually leading to adverse consequence such as (sudden) death (McEwen and Wingfield 2003).

The catfish is known for its sturdiness and transportation conditions described here appear to be tolerated by this species as we did observe recovery and no mortality. It should be noted, however, that fish used in this experiment returned to the farm instead of ending at a slaughterhouse and that conditions at the farm are not similar as those found at slaughterhouses; here, fish would likely be exposed to additional stressors such as adverse water conditions (Schram et al. 2010) and drops in water temperature (van den Burg et al. 2005). Thus, given the fact that the stress axis is already activated in transported catfish, it is entirely conceivable that fish may experience distress from additional stressors at slaughterhouses and suffer from allostatic overload leading to enhanced risk of the (immediate) outbreak of disease, sudden death or (in general) poor welfare. In similar vein, when husbandry conditions on farms are suboptimal, transportation alone may easily impose allostatic overload and thus increased risk of disease, sudden death or (in general) poor welfare.

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