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Recovery from transportation by road of farmed European eel (*Anguilla anguilla*)

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Abstract

The objective of this study was to assess the effects of transportation of marketable eel (0.15 kg) in the Netherlands with respect to welfare. Eels (*Anguilla anguilla)* were obtained from a commercial farm and acclimatized for 7 weeks at the laboratory. Fish were transported according to regular commercial procedures. The animals were placed in water-filled transport tanks on the trailer. Fish density increased from 72 kg m^{-3} (husbandry) to 206 kg m^{-3} (fasting) and was further increased to 270–290 kg m^{-3} during transport. Fish transport lasted 3 h after which the eels were returned to laboratory recirculation systems to measure parameters indicative of stress load, i.e. mortality, plasma cortisol, lactate and non-esterified fatty acids (NEFA) as well as gill morphology. Samples were taken at 0, 6, 24, 48 and 72 h after transport in transported fish and non-transported counterparts (controls). Transportation affected water quality within known tolerable limits. No mortality during or after transport was observed. After 6 h, plasma cortisol levels had returned to baseline. However, energy metabolism had increased suggesting that transportation of eels resulted in an increased energy demand that lasted for at least 72 h in the fasted animals. Thus, it is conceivable that exposure to adverse conditions, prior to stunning/killing, in a slaughterhouse may result in allostatic overload in eel.

Keywords: European eel, stress, transport, welfare, cortisol, *Anguilla anguilla*

Introduction

In aquaculture live fish are transported on farms, between farms and from a farm to a processing facility to slaughter or kill them. In general, transportation of fish comprises the following steps (1) fasting that may range from 24 to 168 h, depending on species and water temperature; (2) crowding, which is followed by capture; (3) loading a transport vehicle; (4) transport while being kept in a tank or closed bag; (5) unloading of fish and; (6) releasing fish in a new environment or handling them to commence slaughter (Dalla Villa, Marahrens, VelardeCalvo, Di Nardo, Kleinschmidt, Fuentes Alvarez, Truar, Di Fede, Otero & Müller-Graf 2009). A limited number of studies show that transportation of live fish may induce strong stress responses that can affect behaviour, physiology and general health of fish over a prolonged period thereafter (Specker & Schreck 1980; Davis & Parker 1986; Schreck, Solazzi, Johnson & Nickelson 1989; Iversen, Finstad & Nilssen1998). Winton (2001) described that handling and crowding, which are part of transportation, influence susceptibility of fish to disease. These studies thus show that transportation of live fish may impair their welfare.

To protect the welfare of animals during transport, the European Council Directive 2006/88/EC 2006/88/EC (2006) was issued. Companies must keep records of all movements of aquaculture animals and products into and out of the site or sites and of mortality of animals during transport. The lack of further requirements for fish in the

Directive can be traced back to the limited available body of research data on transportation of farmed fish as indicated above. However, this limited number of studies has shown that transportation will activate the stress system in fish comparable to what is known in farmed land-based animals (Broom 2005; Warris 1998). This also raises the question whether welfare of farmed fish is affected during transport, as transport leads to a simultaneous exposure of the animals to a variety of stressors in a relatively short period of time. For the interpretation of behavioural and physiological changes as well as general changes in health status, which result from transportation in terms of good or poor welfare, we use the concept of allostasis (Korte, Olivier & Koolhaas 2007; Sterling & Eyer 1988). Allostasis assumes stability through change and the capacity to adapt to a dynamic environment. The allostasis concept holds particular value as a model to discriminate between stress responses which are within the limits of the adaptive capacity of animals and those which are outside these limits and thus compromising animal welfare or not (Korte *et al.* 2007).

At present, data on the effects of transportation on physiology in marketable European eel (*Anguilla anguilla*) are scarce. Together with African catfish (*Clarias gariepinus*) and a hybrid of A. catfish and Vundu catfish (*Heterobranchus longifilis*), European eel comprises the majority of fish species that are transported in the Netherlands for production of food. Within these species, European eel constitutes the most important one with a production of 3200 tons (FEAP 2011) in the Netherlands and 7000 tons in 2010 in Europe (FAO 2012).

Given the importance of European eel for Dutch Aquaculture, we decided to expose this species to a simulated overland transportation to assess how this influences the physiological stress response and how long recovery from transportation may take. Therefore, in our study a commercial transporter drove marketable eels, which were kept in our laboratory, for 3 h (a period representative for regular commercial practice) and then returned to our laboratory. Upon return, the fish were sampled for parameters indicative for activation of the stress system (cortisol; Martinez-Porchas & Martinez-Cordova 2009), changes in energy metabolism as a consequence of fasting- and transport-related stress (glucose, lactate, non-esterified fatty acid (NEFA); Rosen & Spiegelman 2006), changes in general plasma ion-levels as a consequence of fasting- and transport-related stress (osmolality; Kammerer, Cech & Kültz 2010) and changes in gill morphology and histology (e.g. migration of chloride cells towards lamellar regions; Roques, Abbink, Geurds, van de Vis & Flik 2010) as a consequence of fasting- and transport-related stress. One group was sampled directly upon return (0 h), while four other groups were sampled at 6, 24, 48 and 72 h following transport. Controls were held under identical conditions, but were not transported and remained at the laboratory.

Materials and methods

Fish and husbandry conditions

The experiment was conducted with a total of 1300 eels (*Anguilla anguilla*; mixed sexes; 149 ± 50 grams) grown to a normal Dutch slaughter weight at a local eel farm under commercial farming conditions (250 kg m^{-3}, $T = 25°C$). We used, in total, 170 animals for sampling, the remainder was sold by the farmer. Both in pre- and post-fasting sampling, 10 animals were taken. Both transported groups and the control group were sampled at fixed intervals after transport/holding period i.e. at 0, 6, 24, 48 and 72 h after transport/holding period (Fig 1). Every sample point consisted of 10 animals. Transportation was performed in duplicate and therefore the number of animals was finally 20 per sample point. It should be noted that we did not re-use the fish.

At arrival in our facilities, eels were placed at a commercial stocking density (approximately 67 eels per aquarium) in all-glass aquaria with 140 L water (bottom area 90 × 50 cm, height 40 cm; distance from the water level till the glass lid was 9 cm) which were covered with a glass lid. Seventeen aquaria were part of two recirculation systems (total system volume: 2500 L) with biofilters. Fish were housed at a density of 72 kg 1000 L^{-1}, average water temperature of $T = 24 \pm 0.3°C$, an oxygen level of at least 6 mg O_2 L^{-1} and a 12–12 h light–dark cycle (lights on: 06:30 AM). The stocking density is within the range of normal production as is documented by the EFSA (2008). Oxygen, temperature, pH, food, were all according to commercial farming conditions. The experimental procedure (fasting, transport and post-transport observation) was started after an acclimation period of 7 weeks. During acclimation, the fish were manually fed twice daily (09.00 and 17.00), in

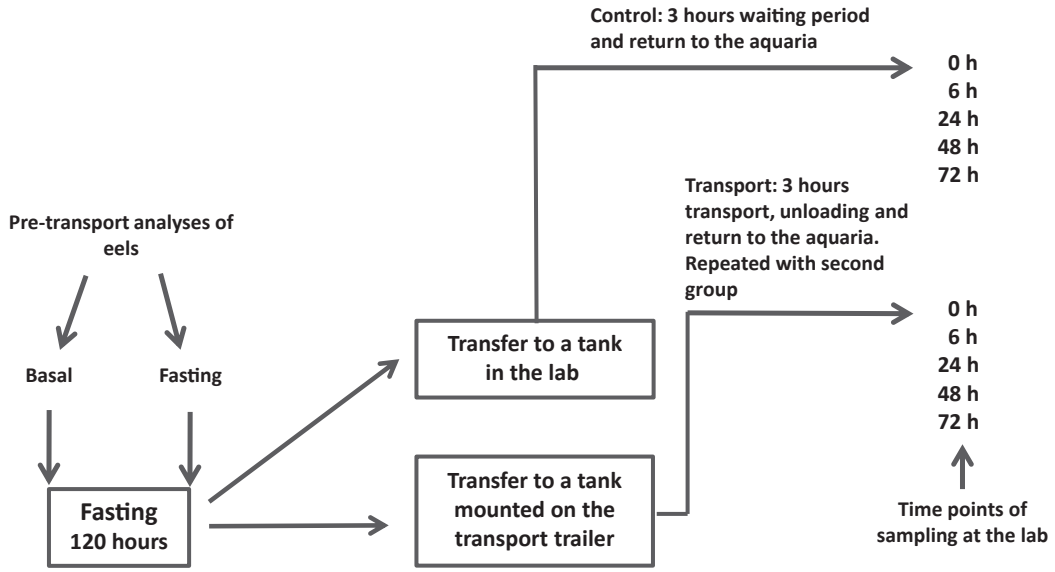

Figure 1 Schematic set-up transport experiment of European eel. Eels were fasted for 120 h after which an experimental group and control group were created. The experimental group was transported for 3 h, subsequently returned to the aquaria facilities and sampled at indicated time points. The control group spent 3 h in a transport tank (not transported), the eels were returned to their aquaria and subsequently sampled at indicated time points. Fish were analysed at time points indicated by arrows.

total 10 g feed day^{-1} kg^{-1} eel. Commercial eel feed was used (Skretting; composition: 47% protein, 14% fat, 7,5% ashes, 2,3% cellulose, 1% phosphor, 5 mg kg^{-1} copper, vitamin E, A, D3). Preceding transport eels were fasted for 120 h. For marketable eels that are cultivated in recirculation systems, this fasting period is necessary for removal of undesirable off-flavours (EFSA 2008). Fasting was performed according to regular commercial farming conditions. After 24 h of food deprivation, water temperature was lowered over 24 h from 24°C to 19°C according to production practice, stocking density was increased to 206 kg 1000 L^{-1} by placing a vertical glass plate in the tank that reduced the volume to 1/3. Increasing the density during fasting by a factor of 3 is customary in eel aquaculture. When water temperature reached 19°C, feed deprivation was continued over the next 96 h. At this point, the fish were ready for the actual transport.

Experimental design

European eel was transported according to general production standards and practices used in the Netherlands. Animals were transported for 3 h at a stocking density of 270–290 kg 1000 L^{-1}. Eels from 5 aquaria were carefully netted and loaded in a polystyrene transport tank. Subsequently, the tank was closed by a wooden lid. This tank (bottom area: 0.95 × 1.05 m, height: 1.00 m) was filled with 150 L water from the recirculation system that contained the eels. During transport, water was oxygenated, via a fenestrated ring on the bottom of the tank, using pure oxygen. Transport was performed by a hauler who checked the oxygen level in the water manually after 1.5 h of transport, using the Hach Lange, HQ 40 multimeter. For approximately 50% of the Dutch haulers this is a standard procedure during eel transport which lasts about 1 min. The route taken was planned beforehand using GPS and represented a normal, average, transport route. After transport, fish were randomly returned to the five glass aquaria, i.e. group composition in each tank after transport was thus different from before transport. Transportation was repeated with eels from five other aquaria. Before transport, water in the transport tank was changed. Another five aquaria were emptied, mixed and placed in a non-transparent plastic 150 L tank covered with a wooden lid to function as control. This tank was also supplied with pure oxygen in the water, but oxygen levels were not measured half-way the holding period as no major changes were expected in the control eels. After 3 h, these eels were returned to the five

aquaria. Post-transport stocking density was 72 kg 1000 L^{-1} for all groups as the glass plates, which had been inserted to increase densities, were removed. The remaining two aquaria were used to analyse basal and fasting conditions before the transport.

Prior to the experiment, 10 fish from one aquarium were taken randomly for sampling (*basal;* Fig 1). Another 10 fish from one aquarium were randomly sampled after 120 h of fasting (*fasting*; Fig 1). Both transported groups and the control group were sampled at fixed intervals after transport/holding period i.e. at 0, 6, 24, 48 and 72 h after transport/holding period (Fig 1). Fish were not fed during the recovery period to avoid adverse water conditions. During sampling, fish were randomly caught and blood and gills were taken ($n = 10$ per aquaria). Also, weights were noted and water samples were analysed.

Fish euthanasia and sample collection

Randomly, fish were removed from their holding tanks by netting and placed within a large water-filled bucket containing 0.1% (v/v) 2-phenoxyethanol (Sigma, St. Louis, MO, USA). Once anaesthetized (within 1 min), fish were removed from the water and blood was drawn after which the fish were immediately killed by transecting the spinal cord right behind the skull. Blood was drawn using heparinized syringes and 1 mL of blood was collected in vials (Eppendorf, 1.5 mL) and immediately put on ice. Subsequently, the vials were centrifuged (1800 *g*, 4°C, 10 min) and blood plasma was separated from blood cells and stored at −20°C until analysis.

Gill tissue was collected and put into 50 mL Greiner tubes containing BOUIN fixative (filtrated; saturated picric acid, saturated formaldehyde (37%), glacial acetic acid (15:5:1 ratio)). The next day samples were dehydrated through steps of increasing ethanol percentages and finally embedded in paraffin. We mounted 7 μm thick tissue sections on gelatinized glass slides and dried.

In addition, we also inspected whether or not mortality occurred in the course of the study.

Water quality

Since the water quality in the tanks in the laboratory was controlled with regard to temperature, pH, ammonia levels, refreshment and oxygenation, the number of water samples taken before and during the experiment was reduced. In the aquaria, water temperature was stable (19°C ± 0.09). Analyses of Total Ammonia Nitrogen (TAN, recalculated to mg $NH_3{}^-N$ L^{-1} by use of the data of Emerson, Russo, Lund & Thurston 1975), Nitrite-N (NO_2), Nitrate-N ($NO_3{}^-$) levels were performed with Tetra test kits (Tetra Werke, Melle, Germany). Temperature, pH and oxygen concentrations were continuously measured by Hach Lange HQ 40 multimeter.

Blood plasma analysis

Cortisol was measured as previously described by Gorissen and colleagues (Gorissen, Bernier, Manuel, de Gelder, Metz, Huising & Flik 2012). Briefly, 96-well microtitre plates were coated with mouse cortisol antibodies in a coating buffer. Plates were cleared of coating buffer and washed with a wash buffer before blocking possible a-specific binding sites with a 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 proper wells. After the incubation period, wells were cleared and washed before scintillation liquid was added. Activity within the wells was measured using a β-counter.

Glucose, NEFA and lactate were measured using commercially available kits (Wako Diagnostics, Richmond, VA, USA). Plasma osmolality (sample volumes: 50 μL) was measured with a cryoscopicosmometer (Osmomat 030, Gonotec, Germany). Deionized water (0 mOsmol kg^{-1}) and a standard solution (300 mOsmol kg^{-1}) were used as reference. Plasma sodium and plasma chloride were photometric determined with the Sodium rapid kit and Chloridliquicolor kit of Human (Human Diagnostics, Wiesbaden, Germany).

Gill histology

After paraffin removal from the collected gill tissue samples, sections were either placed in an alcian blue solution to stain mucus producing cells or 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 sections incubated overnight with antibodies against Na/K-ATPase (final dilution 1:300) to stain chloride cells. Donkey anti-rabbit was used as secondary antibody at a dilution of 1:200. Sections were subsequently incubated with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA,

USA). Staining was performed in 0.025% (w/v) 3,3′-diaminobenzidine (DAB) and 0.0005% (v/v) H_2O_2.

Statistics

Data were checked for normal distribution and statistical analysis was performed in Graphpad Prism 5 (Graphpad Software, La Jolla, CA, USA) using Kruskal–Wallis (when not normally distributed) or a One-Way ANOVA (when normally distributed) to test for significance between either experimental or control groups over time; time points are independent samples. To test for significance between an experimental and control group at specific time points, a Mann–Whitney *U*-test (when not normally distributed), unpaired *t*-test or unpaired *t*-test with Welch's correction were used (when normally distributed and depending on sample distribution). Significance was set at $P \leq 0.05$. Data are expressed as means ± 1 standard deviation (SD). For all figures: groups with the same letters are not significantly different.

Results

Water quality

Water quality was closely monitored during the experiment. Data are shown in Table 1; pH-values were adjusted where necessary to represent farming conditions (see Table 1). During fasting, the pH-value was down-regulated, when it was higher than 7.9 using nitric acid (1:10 diluted). The pH-values fluctuated in the course of the experiment between 6.9 and 7.4. Transport tanks were filled with water from the recirculation system, which contained a dissolved oxygen concentration of 5.50 and 6.36 mg L^{-1} at the beginning of transport. At the end of the transport, i.e. after 3 h, oxygen concentrations were between 11.96 and 10 mg L^{-1}. After transfer of the control group into the tank, the oxygen concentration decreased to 3.0 mg L^{-1}. During the waiting period, the oxygen concentration started to increase to 4.46 mg L^{-1} due to pure oxygen supply in the water. Water temperature fluctuated during transport between 17°C and 21°C. In the control group, temperature dropped from 21.4°C to 20.0°C over 3 h. TAN was low in the aquaria throughout the experiment *i.e.*, between 0.21 and 1.2 mg $(NH_3+NH_4^+)$-N L^{-1}. Before fasting, nitrite levels varied between 0 and 1.5 mg NO_2^--N L^{-1}. After transport, nitrite-N in the water varied between <0.091 and 0.45 mg NO_2^--N L^{-1}. Prior to fasting, nitrate-N levels in the water fluctuated between 5 and 57.5 mg NO_3^--N L^{-1}. Directly after transport, nitrate levels were 2.8 mg NO_3^--N L^{-1} in all groups.

Table 1 Water quality during transport and the recovery period following transport

	pH			O_2 (mg L^{-1})			Temperature (°C)		
	Control	Batch 1	Batch 2	Control	Batch 1	Batch 2	Control	Batch 1	Batch 2
Post fasting	7.67	7.67	7.65	9.3	9.3	9.3	18.4	18.4	18.6
Transport tank (begin of transport)	7.67	7.43	7.62	2.95	5.5	6.36	21.4	19.5	19.3
Transport tank (after transport)	6.94	7.91	7.23	4.46	11.96	10	20	18.6	17.1
(Time after transport)									
0	7.68	7.73	7.73	9.83	11.16	11.16	18.8	18.6	18.6
24	7.82	7.7	7.7	7.7	6.8	6.8	18.9	18.9	18.9
48	7.85	7.73	7.73	7.8	6.7	6.7	19.1	19	19
72	7.81	7.74	7.74	8.3	6.2	6.2	19.4	19.2	19.2
	TAN $(NH_3+NH_4^+)$-N mg L^{-1})			NO_2^--N (mg L^{-1})			NO_3^--N (mg L^{-1})		
	Control	Batch 1	Batch 2	Control	Batch 1	Batch 2	Control	Batch 1	Batch 2
Post fasting	0.2059	0.2059	0.2059						
Transport tank (begin of transport)	0.2059	0.2059	0.2059						
Transport tank (after transport)	0.2059	0.2059	1.2353		0.091	0.45	2.8	2.8	2.8
(Time after transport)									
0	n.d.	0.2059	0.2059	n.d.	n.d.	0.24			
24	n.d.			0.091	0.091	0.091			
48	n.d.	n.d.	n.d.	0.091	0.091	0.091			
72	n.d.	n.d.	n.d.	0.091	0.091	0.091			

Empty cells: not measured.
n.d., not determined.

Stress axis

While we sampled 10 (control) to 20 (transport, in duplicate) animals per time point, we did not obtain data for all animals for the following two reasons, (1) due to haemolyses we had to discard the sample, (2) the volumes of plasma samples were too small to measure all parameters. Therefore, in the bars of the figures the numbers are lower than 10 (control) or 20 (transport groups).

Basal plasma cortisol levels were low: 1.0 ± 0.6 ng mL^{-1}. Also, values after 120 h fasting were low: 1.6 ± 1.2 ng mL^{-1}. Transporting European eel by a hauler led to a significant increase of plasma cortisol levels immediately after transport (levels at 0 h; Fig 2; 10.2 ± 5.73 ng mL^{-1}) compared with basal levels. In contrast, for the control no differences were found between levels immediately after transport (levels at 0 h; Fig 2; 4.4 ± 3.3 ng mL^{-1}) and basal levels. Furthermore, blood plasma cortisol levels in transported fish were significantly (Mann–whitney *U*; $P = 0.0145$) higher compared with levels in control fish, immediately after transport (levels at 0 h; Fig 2). After 6 h, plasma cortisol levels were not significantly different from basal levels in the transported or control group, neither was there a significant difference between the transported and control group. In the course of the recovery period (72 h in total), plasma cortisol levels in the transported eels fluctuated between 8.5 ± 6.4 ng mL^{-1} at 6 h and 19.3 ± 10.7 ng mL^{-1} at 24 h after transport. Both in the transported fish and control fish, we observed a gradual increase of plasma cortisol levels compared with baseline levels as the recovery period continued, starting at 24 h of the recovery period and lasting up to 72 h. At 24, 48 or 72 h, no significant differences between transported and control fish were found. We did not observe any mortality in the course of the experiment.

Energy metabolism

Glucose

Plasma glucose analyses showed baseline plasma glucose levels of 5.8 ± 2.5 mmol L^{-1} (Fig 3). These levels were slightly, but not significantly, decreased after 120 h of fasting: 4.3 ± 1.8 mmol L^{-1} (One-Way ANOVA; $P = 0.15$). Directly after transport, plasma glucose levels were significantly increased (0 h; 7.3 ± 0.5 mmol L^{-1}; One-Way ANOVA; $P = 0.0013$) compared with after fasting levels, but not compared with baseline levels. These levels returned to fasting levels at 24 h (5.1 ± 1.9 mmol L^{-1}; One-Way ANOVA;

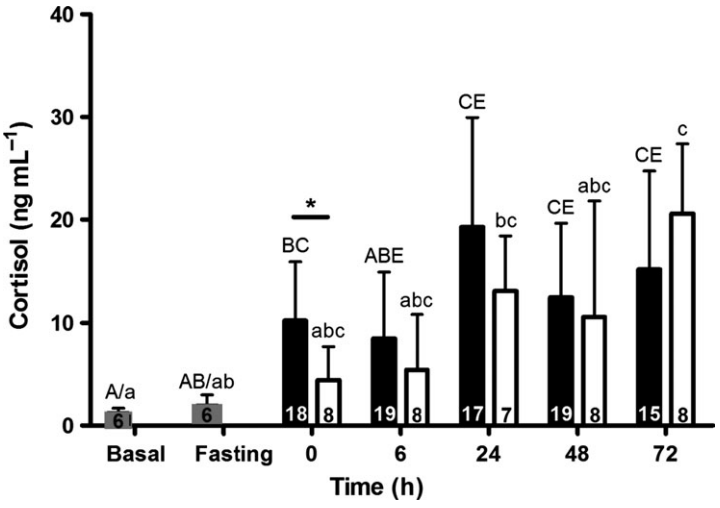

Figure 2 Effects of plasma cortisol levels in European eels exposed to transportation. Analyses occurred before the experiment (basal), directly after the fasting period of 120 h but before transport (fasting), while sampling points post transport were: 0, 6, 24, 48 and 72 h. Grey bars indicate sampling prior to loading of the truck, black bars indicate plasma cortisol titres of transported groups and white bars of the control group. Number of subjects per sampling point is indicated in the bars. Significance was accepted at $P < 0.05$ (*) and values are expressed as mean + 1 standard deviation. Letters indicate significance over time within control (lowercase letters) or transported (capital letters) groups. Groups with the same letter were not significantly different from each other.

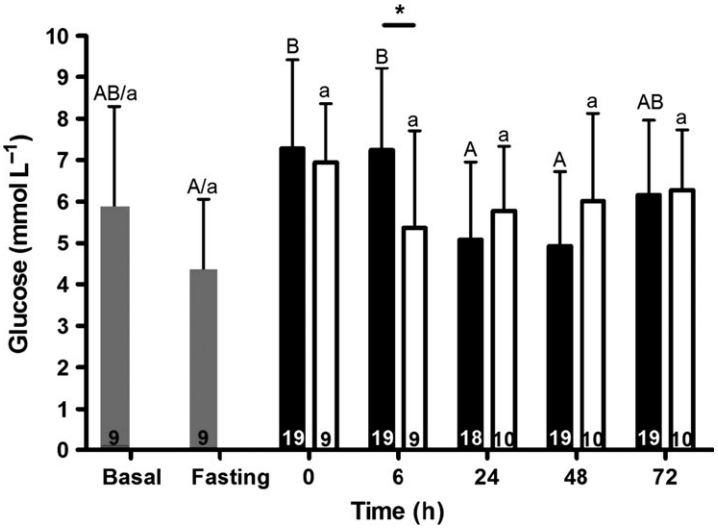

Figure 3 Effects of transport at plasma glucose levels in European eels. Analyses occurred before the experiment (basal), directly after the fasting period of 120 hours but before transport (fasting), while sampling points post transport were: 0, 6, 24, 48 and 72 h. Grey bars indicate sampling prior to loading of the truck, black bars indicate plasma glucose titres of transported groups and white bars of the control group. Number of subjects per sampling point is indicated in the bars. Significance was accepted at $P < 0.05$ (*) and values are expressed as mean + 1 standard deviation. Letters indicate significance over time within control (lowercase letters) or transported (capital letters) groups. Groups with the same letter were not significantly different from each other.

$P = 0.0008$) after transport. Hereafter, they were similar to baseline and fasting levels. In the control group (basal, fasting), plasma glucose levels did not change significantly after the waiting period (0, 6, 24, 48, 72 h). After 6 h (7.2 ± 0.5 mmol L^{-1}), plasma glucose levels in the transported fish were significantly higher than in the control fish (5.4 ± 2.3 mmol L^{-1}; unpaired *t*-test; $P = 0.0361$).

NEFA

Analyses of plasma NEFA levels showed baseline plasma NEFA levels of 0.34 ± 0.07 mmol L^{-1} (Fig 4). These values changed little after 120 h of fasting: 0.30 ± 0.05 mmol L^{-1}. There was a significant increase in plasma NEFA levels after 48 h (0.39 ± 0.08 mmol L^{-1}; One-Way ANOVA; $P = 0.0083$) and 72 h (0.42 ± 0.06 mmol L^{-1}; One-Way ANOVA; $P = 0.0007$) after transport, compared with post-fasting levels but not compared with baseline levels. The control group differed significantly from baseline levels at 0 h (Kruskal–Wallis; $P = 0.0079$) and 6 h (Kruskal–Wallis; $P = 0.0042$) of the recovery period. We observed significant differences between control and transported groups throughout the experiment (Unpaired *t*-test; 0, $P = 0.0079$; 6, 24, 48, 72 h,) (Fig 4: (Unpaired *t*-test; 0 h, transport 0.31 ± 0.08, control 0.22 ± 0.08, $P = 0.0079$; 6 h, transport 0.38 ± 0.06, control 0.22 ± 0.07, $P < 0.0001$; 24 h, transport 0.36 ± 0.08, control 0.27 ± 0.04, $P = 0.0012$; 48 h, transport 0.39 ± 0.08, control 0.32 ± 0.10, $P = 0.0334$; 72 h, transport 0.42 ± 0.08, control 0.29 ± 0.06, $P < 0.0001$).

Lactate

Analyses of plasma lactate levels revealed basal plasma lactate levels of 11.18 ± 6.6 mmol L^{-1}, while post-fasting plasma lactate levels were 9.6 ± 3.6 mmol L^{-1} (Fig 5). Plasma lactate levels decreased significantly 6 after transport (2.72 ± 1.1 mmol L^{-1}), compared with baseline levels (Kruskal–Wallis; $P < 0.0001$), after fasting levels ($P < 0.0001$) and 0 h post-transport levels (6.9 ± 2.9 mmol L^{-1}; $P < 0.0001$; Fig 5). Plasma lactate levels remained significantly lower than baseline levels and post-fasting levels throughout the experiment for the transported group. For the control group, plasma lactate levels decreased significantly at 6 h (2.99 ± 2.0 mmol L^{-1}) compared with baseline levels (Kruskal–Wallis; $P = 0.0021$) and after fasting levels ($P = 0.0012$).

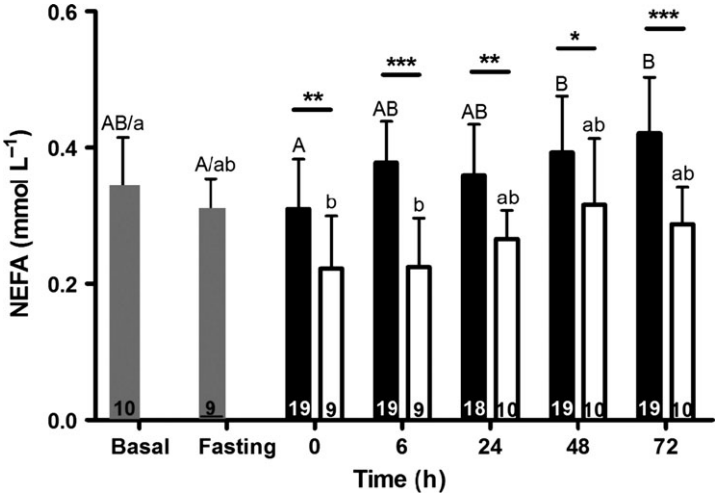

Figure 4 The effect of transport on non-esterified fatty acid levels in plasma of the European eel. Analyses occurred before the experiment (basal), directly after the fasting period of 120 h but before transport (fasting), while sampling points post transport were: 0, 6, 24, 48 and 72 h. Grey bars indicate sampling prior of loading of the truck, black bars indicate plasma NEFA titres of transported groups and white bars of the control group. Number of subjects is indicated in the bars. Significance was accepted at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) and values are expressed as mean + 1 standard deviation. Letters indicate significance over time within control (lowercase letters) or transported (capital letters) groups. Groups with the same letter were not significantly different from each other.

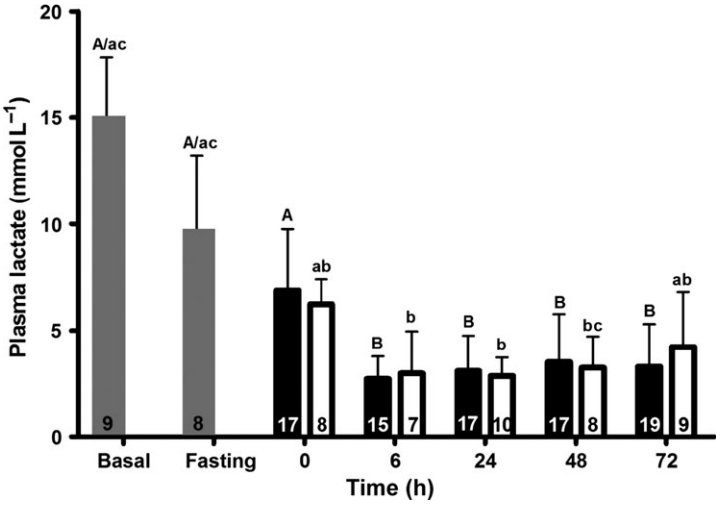

Figure 5 The effect of transport on lactate levels in the blood plasma of the European eel. Analyses occurred before the experiment (basal), directly after the fasting period of 120 h but before transport (fasting), while sampling points post transport were: 0, 6, 24, 48 and 72 h. Grey bars indicate sampling prior to loading of the truck, black bars indicate plasma lactate titres of transported groups and white bars of the control group. Number of subjects per sampling point is indicated in the bars. Values are expressed as mean + 1 standard deviation. Letters indicate significance over time within control (lowercase letters) or transported (capital letters) groups. Groups with the same letter were not significantly different from each other.

Osmolality

We did observe significant differences (24 h, 0.26 ± 0.04 mOsmol kg^{-1} Kruskal–Wallis; $P = 0.0019$; 48 h, 0.24 ± 0.04 mOsmol kg^{-1}; $P = 0.0002$) in the transported group, compared with 0 h post transport (Fig 6). No differences were detected in the control group. Plasma sodium levels ranged from 142.0 ± 15.6 mmol L^{-1} to 152.4 ± 5.2 mmol L^{-1}

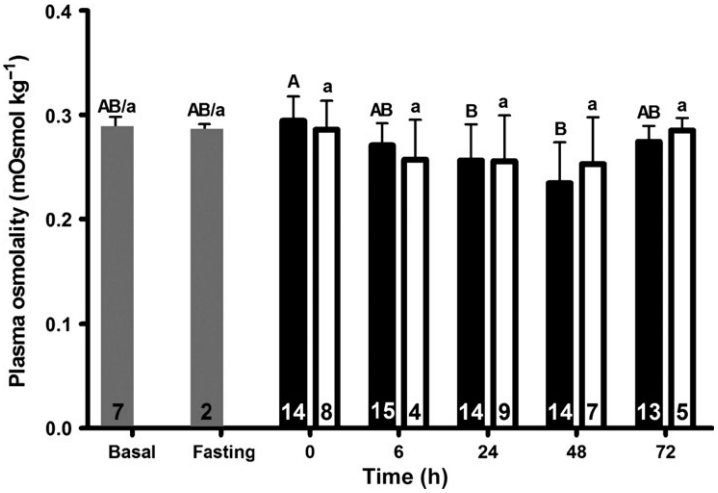

Figure 6 The effect of transport on plasma osmolality of the European eel. Analyses occurred before the experiment (basal), directly after the fasting period of 120 h but before transport (fasting), while sampling points post transport were: 0, 6, 24, 48 and 72 h. Grey bars indicate sampling prior to loading of the truck, black bars indicate plasma osmolality of transported groups and white bars of the control group. Number of subjects per sampling point is indicated in the bars. Values are expressed as mean + 1 standard deviation. Letters indicate significance over time within control (lowercase letters) or transported (capital letters) groups. Groups with the same letter were not significantly different from each other.

and plasma chloride from 73.0 ± 23.0 mmol L^{-1} to 125.0 ± 33.9 mmol L^{-1}. For both sodium and chloride plasma levels no significant changes occurred due to transportation, compared to basal and fasting levels.

Gill histology

Gill histology was done on a selected sample ($n = 4$) of animals since the water quality and osmolality did not significantly differ between before and after transport. Transport showed no effect on the frequency of mucous-containing cells: transported (116.5 ± 45.0; $n = 4$) versus control (171.6 ± 72.3; $n = 4$) versus basal (154.3 ± 28.7; $n = 4$). Also, no significant migration of chloride cells towards lamellar regions was observed. No deterioration of gill morphology was observed.

Discussion

Our study showed that transportation of eels by a hauler affected plasma cortisol levels mildly and transiently as 6 h post transport no significant difference could be detected between the transported groups and the control group. Nevertheless, the release of cortisol resulted in a significant change in the energy metabolism of the transported eels throughout the experiment that lasted at least 72 h post transport, as judged from analysis of NEFA levels in plasma. The duration of the increase in the metabolism suggests that transportation of eels resulted in a strong physiological response in the fasted animal. Levels of glucose were significantly increased until 6 h post transport. Lactate in blood plasma decreased and remained low throughout the experiment. Plasma osmolality was not affected by transport. During transportation, water quality did not deteriorate and gill physiology and morphology did not change. Transportation of the eels did not result in mortality, under conditions used.

Water quality

The oxygen level, pH, TAN, nitrite-N and nitrate-N in the water never reached critical levels, indicating that the stocking density used did not lead to an unacceptable water quality during transportation. The decrease in water temperature during transport was no surprise since the transport vehicle had no thermoregulation, only insulation. Increasing oxygen concentration during transport was likely due to oxygenation. The levels of TAN (recalculated to mg NH_3-N L^{-1} with use of the

database published by Emerson *et al.* 1975), nitrite-N, nitrate-N and pH during transport of the eels with a stocking density of 270–290 kg 1000 L^{-1} did not exceed threshold values for these substances. The thresholds are 0.058 mg L^{-1} for NH_3-N (20 mg L^{-1} for nitrite-N (Kamstra & Span 1996) (Yamagata & Niwa 1979), 100 mg L^{-1} for nitrate-N (Kamstra & van der Heul 1998) and an acidity level above pH 5 (Kamstra & van der Heul 1998). Analyses of the oxygen level showed a decrease in oxygen in water (3 mg L^{-1}) of the control group after transfer in the new tank. Cruz-Neto and Steffensen (1997) showed that European eel acclimatized to 25°C decreased their oxygen consumption when oxygen levels in the water are below 1.9 mg L^{-1}. The oxygen levels in our experiment were higher, at lowest 3 mg L^{-1}, and were kept at a lower temperature (19°C) than this, and therefore we assume that the metabolism of the eels was not detrimentally affected. Post transport, the oxygen concentration in the water dropped in the first 24 h. This is likely due to the handling of the animals.

Cortisol

It appeared that plasma cortisol levels in the eels that were transported were only mildly affected by this process, under the conditions used. There is on average a 6.4 fold (from 1.6 to 10.2 ng mL^{-1}) increase in the cortisol level, compared with the fasted animals. A plasma level of 10.2 ng mL^{-1} on average is (still) low, as for other fish species like carp or salmon, is it known that this may reflect a normal baseline (Metz, Huising, Meek, Taverne-Thiele, Wendelaar Bonga & Flik 2004; Fast, Hosoya, Johnson & Afonso 2008). However, when one notices the increased energy metabolism, as shown by the NEFA data, it is likely that we missed the window during which plasma cortisol levels in the eels increased to a higher value, as Gollock, Kennedy, Quabius and Brown (2004) showed that 4 h after exposure to a stressor plasma cortisol returned to basal levels. We, therefore, assume that in our study this window occurred during transport that lasted for 3 h. Analysis during this period was not an option, as this would have interrupted the experiment. In addition, also Vijayan and Pereira (1994) observed that in rainbow trout exposed to acute stress the window during which plasma cortisol increased and returned to basal levels was 6 h. Lack of nutrition is a plausible explanation for the steady rise in plasma cortisol which started at 24 h post transport. At the end of the experiment, the fish have been deprived of food over 192 h which can evoke metabolic changes directed by cortisol. It is known that the stress axis is intertwined with food intake (see reviews of Bernier & Peter 2001; Wendelaar Bonga 1997). It seems that for a migrating fish like the European eel analyses of only plasma cortisol is not a good biomarker for stress and additional measurements are required.

Glucose

Even though cortisol reflects normal homeostatic variation, there are enduring changes to the metabolic system. Average glucose levels were ranging from 4 to 7.5 mmol L^{-1}, which is very similar to other experiments with European eels (Teles & Maria 2003). Glucose in the blood increased significantly during the first 6 h after transport compared with baseline or post-fasting levels but returns within 24 h to basal. Hyperglycaemia in the first hours after transport can indicate that the fish experience stress (Arends, Mancera, Muñol, Wendelaar Bonga & Flik 1999) and we might even have missed the highest production within 6 h post stress (Vijayan & Pereira 1994). It has already been shown in *Anguilla japonica* that injections with cortisol can increase blood glucose (Chan & Woo 1978). Similar effects have been shown in experiments with *Anguilla rostrata* (Butler 1968). Glucose is an important driver of metabolism and certain tissues (involved during stress) may rely primarily on glucose (for example brain, heart, blood cells, and gills; Mommsen 1986). However, the quick return of glucose to basal indicates that the immediate stress response on transport is not long lasting.

The lasting decrease in plasma lactate indicates that transport has a more enduring effect than initially was concluded from the cortisol and plasma glucose data. These data suggest that the fish have the need for additional energy as lactate can be converted to pyruvate and used in the Krebs cycle. It is known that lactate can be used as an energy source in tissues (involved during stress) like gills, kidney, liver and brain (rev. Soengas, Sangio-Alvarellos, Laiz-Carrión & Mancera 2007; Soengas, Andrés & Strong 1998).

NEFA

There is a significant difference in plasma NEFA levels between all transported and control groups. This suggests a change in the energy metabolism, as we also observed a descent in plasma lactate levels after transportation. Due to this change, the eels were able to cope with the increased energy demand, which is probably needed to fuel cellular processes. Plasma NEFA levels decreased in the control groups at 0 and 6 h post transport, compared with the baseline level. This may be caused by the drop in temperature at the start of fasting (24 to 19°C). However in the transported groups, plasma NEFA levels did not change compared with baseline levels. This could be due to the additional energy needed because of stress induced by transportation.

Osmolality

No chances in plasma osmolality and in also plasma sodium and chloride levels were found. It is known that stress can change the permeability of the gill membranes (Dang, Balm, Flik, Wendelaar Bonga & Lock 2000), thereby potentially influencing the osmolality of the blood. The increase of cortisol does apparently not change gill permeability and thereby the osmolality. This is in accordance with the gill physiology, where no differences were observed in the amount of mucous cells between the groups.

Biological relevance

Elevated plasma cortisol is a primary stress response and a strong indicator of stress in animals. Plasma cortisol levels in eel were only very mildly affected by transport, as after a recovery period of 6 h baseline levels were obtained. However, as shown by Gollock *et al.* (2004) in European eels cortisol is not necessarily a good readout to address stress or welfare. The duration (72 h) of the increased metabolism suggests that transportation of eels resulted in an increased energy demand in the fasted animal. Following the 3 h-transport, differences in cortisol levels were observed between eels as indicated by the larger SD values in Fig 2. This may suggest differences in coping style between subjects (Koolhaas, Korte, De Boer, Van Der Vegt, Van Reenen, Hopster, De Jong, Ruis & Blokhuis 1999). This warrants further studies as these differences may be associated with differences in allostatic load (Koolhaas *et al.* 1999). The adverse effect of a recovery period of more than 72 h post transport was scored as 5 on a scale of 1 to 5 by Dalla Villa *et al.* (2009). Also, it is conceivable that exposure to adverse conditions, prior to stunning/killing, in a slaughterhouse may result in allostatic overload in eel. This has been shown in a study by Bogdan and Waluga (1980), who reported high mortality among elvers especially on second day after transport, mainly by pathogenic causes. This indicates that transport is a potential hazard for this species, as an allostatic overload may occur.

Conclusion

Here, we present evidence that transport of European eel under these conditions seems to be tolerated by the animals as indicated by the physiological parameters, that we measured. Transportation at a density of 270–290 kg eels 1000 L^{-1} affected water quality within known tolerable limits. Nevertheless, our study indicates that the fish were affected for longer period than is revealed by the plasma cortisol titres only, as shown by the plasma NEFA levels.

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