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Stress and disease resilience differences related to emergence time for first feeding in farmed rainbow trout (*Oncorhynchus mykiss*)

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Summary statement

The neuroendocrine response to acute stress and repeated stress of farmed juvenile rainbow trout is related to their individual time of emergence, but has no influence in their performance in captivity.

ABSTRACT

Salmonid individuals show a relatively high variability in the time required to abandon the gravel nest where they hatch, the so-called “emergence time”. Different behavioral and physiological traits have been shown to be associated to that emergence time in wild salmonids. In general, early- and late-emerging fish have traits resembling those of proactive and reactive stress coping styles, respectively. Proactive fish are considered to be more resilient to stress and probably to disease, so it was hypothesized that fish with different emergence time have different ability to resist repeated episodes of stress without suffering deleterious effects on their welfare or health status. In this study, rainbow trout eyed eggs were hatched and larvae were fractionated according to their emergence time (Early fraction: first 20 % of fish to emerge; Intermediate fraction: mid 20 %; Late fraction: last 20 %). When the fish were four months old, part of the fish were exposed to a daily repeated stress protocol for 15 days. The next day, both naïve and repeatedly-stressed fish were exposed to an acute stress challenge. Different plasma (cortisol, glucose, lactate) as well as CNS (serotonergic activity) stress markers were assessed to evaluate the stress resilience of the different fractions. Furthermore, an intraperitoneal infection challenge with *Flavobacterium psychrophilum* was carried out to assess the disease resilience of the different emergence fractions. Altogether, the results showed that fish from different fractions displayed different activation of the hypothalamus-pituitary-interrenal axis, pointing to a higher stress resilience in the fish with shorter emergence times. However, those differences were not reflected in the ability of the different fractions to grow and perform well in terms of growth, or in the ability to overcome the infection with the bacteria, which was similar for all the emergence fractions. This suggests that discriminating fish according to emergence time would probably have little effect in improving the performance and the welfare of farmed fish.

List of Symbols and Abbreviations

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: serotonin

ANOVA: analysis of variance

BA: blood agar

BSC: brain-sympathetic-chromaffin

CFU: colony forming units

CNS: central nervous system

DDF: day-degrees post-fertilization

DNA: deoxyribonucleic acid

HPI: hypothalamus-pituitary-interrenal

HPLC-EC: high performance liquid chromatography with electrochemical detection

IP: intraperitoneal

MALDI: matrix-assisted laser desorption/ionization

MS-222: 3-aminobenzoic acid ethyl ester

PCR: polymerase chain reaction

RS: repeated stress

RTFS: rainbow trout fry syndrome

SCS: stress coping style

s.d.: standard deviation

s.e.m.: standard error of mean

TOF: time-of-flight mass spectrometer

TPS: time post-stress

TYES: tryptone yeast extract salts

INTRODUCTION

Salmonid fish are lithophilic spawners. During the reproductive season, salmonid females deposit the eggs over gravel or cobble substrates after digging a nest (Kondolf, 2000; Sternecker and Geist, 2010). After fertilization, the eggs develop in the gravel interstices until they hatch, and then the fish larvae remain there for some time until their yolk sac is consumed or almost consumed. At this point, salmonid larvae develop a typical swim-up

behavior to abandon the gravel substrate looking for new feeding territories. The timing for this emergence behavior is relatively variable for each individual and it has been reported that it could differ several weeks for fish from the same batch of fertilized eggs (Brännäs, 1995; Garcia de Leaniz et al., 1993).

The time of emergence has been correlated in salmonids to the individual stress coping style (SCS) (Andersson et al., 2013; Metcalfe and Thorpe, 1992; Vaz-Serrano et al., 2011), i.e. the set of physiological and behavioral characteristics of the animal response to challenging or hazardous conditions (Koolhaas et al., 1999). Early emergent individuals have been shown to be bolder, to have a higher probability to become dominant, and to have higher metabolic rates than late-emerging individuals (Andersson et al., 2013; Metcalfe and Thorpe, 1992; Vaz-Serrano et al., 2011), differences that are usually found between fish of proactive and reactive SCS, respectively (Castanheira et al., 2017). Besides, it is well established that fish of proactive and reactive SCS differ in their neuroendocrine and behavioral response to different stressors (Castanheira et al., 2017; Schjolden et al., 2005, 2006; Winberg et al., 2016). As in other vertebrates, the neuroendocrine response to stress in fish is mediated through two different neuroendocrine axes which are responsible for the synthesis and release of the so-called stress hormones (catecholamines and corticosteroids). Thus, upon stress exposure, both the hypothalamus-pituitary-interrenal cells (HPI), and the brain-sympathetic-chromaffin (BSC) axes are activated, resulting in the release of cortisol (main glucocorticoid hormone in teleost fish) and adrenaline and noradrenaline, respectively (Gesto et al., 2013; Wendelaar Bonga, 1997). In proactive fish, the HPI axis is usually activated to a lower extent upon stress exposure, resulting in the release of lower amounts of cortisol into the circulation. On the other hand, the BSC axis is generally activated to a larger extent in proactive fish, resulting in the release of larger amounts of catecholamines than in reactive fish (Castanheira et al., 2017; Schjolden et al., 2006).

The link between emergence time and SCS, together with the SCS-related differences in the fish stress response, raises the question about whether emergence time can have an influence in the individual stress resilience, i.e. the capability of the fish to cope with potential stressors without relevant consequences to normal physiology and behavior. In many cases, stress can also have important deleterious effects in the immune function in fish and other vertebrates, and therefore, stress resilience is linked to disease resilience (Pickering and Pottinger, 1989; Wendelaar Bonga, 1997). In this regard, discriminating fish larvae according to their emergence time could be a potent selection tool to select fish that are more naturally robust toward stress and disease. This selection would have important advantages for fish farming, which could benefit from

the selection of more robust fish against typical and inherent stressors that occur in aquaculture facilities, such as handling, high stocking density or changes in water quality, among others.

In the current study we tested the hypothesis that fish emerging earlier have different resilience to stress and disease than fish that emerge later. Therefore, a stress-resilience experiment was performed with four month-old fish, in which the acute stress response of the fish from the different fractions was evaluated. Besides, the influence of a previous history of repeated stress events on the acute stress response was also assessed. The fish stress response was assessed through plasma stress markers (cortisol, glucose, lactate), as well as forebrain serotonergic activity. In addition, an infection challenge with *Flavobacterium psychrophilum* was carried out to assess the resilience of the different fractions against the rainbow trout fry syndrome (RTFS), one of the most devastating diseases among cultured rainbow trout in Denmark.

MATERIALS AND METHODS

Animals and housing conditions

Approximately 15,000 rainbow trout (*Oncorhynchus mykiss*) eyed eggs (mixed sex) were purchased from a local organic trout hatchery (Piledal Dambrug, Vejle, Denmark) and transferred to the facilities of the Technical University of Denmark (DTU) at the North Sea Science Park in Hirtshals, Denmark. The eggs were incubated at 10 °C under a current of oxygen-saturated water. After hatching, larvae were moved to artificial gravel nests, which contained white golf balls (43 mm diameter) simulating natural gravel. The artificial nests were used as a screening device to separate fish according to their emergence time, taking advantage of the typical swimming behavior the larvae develop when they have almost consumed their yolk sac (see Vaz-Serrano et al., 2011, for a description of the screening device). The emergence behavior started at around 515 day-degrees post-fertilization (DDF). For the duration of the swim up behavior, larvae were sequentially removed from the container, counted, classified according to emergence time and moved to a new facility. Three different fractions were retained according to their emergence time in order to obtain groups clearly differing in this regard: an early fraction consisting of the 20 % of fish that emerged first; an intermediate fraction, consisting of the 20 % of the fish with intermediate emergence time; and a late fraction consisting of the 20 % of the fish that emerged last. The remaining 40 % of the larvae were not used in the experiments. Also, a small percentage of the larvae (< 1 %) had not emerged from the artificial substrate by the time the emergence process was terminated; those fish were neither used in the experiments. The total duration of the emergence period was 9 days at 10 °C, in accordance to a fractionation procedure reported before in rainbow trout (Andersson et al.,

2013). Each emergence fraction was reared in separate tanks at 12 °C for three months until the beginning of the experiments described below.

The use of fish in this study complied with Danish and EU legislation (Directive 2010/63/EU) on animal experimentation and was approved by the Animal Welfare committee of DTU Aqua. The infection challenge done at DTU Vet was performed under the license number 2012-15-2934-00629, issued by the Danish authorities within the field.

Stress resilience experiment

Three months after hatching, the fish had an average mass of 2.3 ± 1.1 g (mean ± s.d.), and there were no differences in mass, fork length or condition factor among the emergence fractions. Fish were then distributed in 600 L tanks; three tanks were used per each of the emergence fractions (Early, Intermediate or Late) for a total of 9 experimental tanks (assigned randomly). In each of those tanks, 500 fish of the corresponding fraction were allocated, making two groups of 250 fish each by using two plastic 48 L aquaria (with a metal grid at the bottom allowing for passage of water) partially submerged in the 600 L tank. Thus, a total of 18 aquaria, with 250 fish each, were used in the experiment. The water (recirculating) was kept at 16 °C and water quality parameters (NO_3^-, NO_2^-, NH_3/NH_4^+, pH, O_2 saturation) were controlled every other day. The photoperiod was kept at 14:10 (L:D). Fish were daily fed commercial feed (3.5 % fish mass day^{-1}; EFICO E 920, Biomar, Brande, Denmark) by means of belt feeders. Fish were kept in those conditions for four weeks before the start of the stress resilience experiment. During the experiment, the fish were exposed for 15 days to a repeated daily stressor, always at the same time of the day. The daily stressor consisted in lifting the plastic aquaria out of the water, exposing the fish to air (acute air exposure). Fish were exposed to air for 45 seconds, returned to the water for 15 seconds, and exposed again to air for 45 seconds. Within each pair of aquaria, one of the aquaria was exposed to the daily stressor while the other served as control and was not exposed. This protocol was applied in the morning and one hour after the daily stress, the fish were provided with feed. After the 15 day period, all fish were exposed next morning to an acute crowding stressor by lifting the plastic aquaria and leaving only a 4 cm water layer (stocking density of 200 kg m^{-3}). The crowding stress lasted for 30 min and then the water level was returned to normal. Fish were sampled (see below) at 0 (just before the crowding stress – controls), and at 1 h, 2 h, 4 h and 8 h after stress. Fish groups not exposed to the repeated stress protocol served as controls for the influence of previous stressors on the acute stress response of the fish. Fifteen fish per fraction and treatment (five from each aquarium) were sampled at each time point.

During the sampling of each aquarium, five fish were netted and deeply anesthetized together in a benzocaine solution (200 mg L^{-1}). Fish became anesthetized in 30 s and blood was then rapidly collected from caudal vessels with ammonium-heparinized syringes. Then, the fish was decapitated and the head immediately frozen on dry ice and later stored at -80 °C. The sampling of each batch of 5 fish took approximately 4 min. The blood samples of each batch of fish were immediately centrifuged and the plasma was stored at -80 °C for subsequent analyses of cortisol, glucose and lactate.

Flavobacterium psychrophilum challenge

A *Flavobacterium psychrophilum* challenge was chosen because of its relevance for European trout aquaculture. RTFS is one of the most prominent fry diseases requiring antibiotic treatment in European trout farms (Jensen et al., 2003). Selecting fish more naturally resilient against this disease is of key importance, especially for organic trout aquaculture, in which medicating fish is only allowed within very strict limits.

Challenge strain

The *Flavobacterium psychrophilum* strain used for the challenges was a well-characterized Danish strain 950106-1/1 (serotype Fd, ribotype A, 3.3 kb plasmid, virulent) (Madsen and Dalsgaard, 1999, 2000).

The strain was stored at -80 °C in tryptone yeast extract salts (TYES) media (Holt et al., 1993) with 15 to 20 % glycerol and was subcultured in agitated cultures at 15 °C. Strains were taken directly from -80 °C and incubated in TYES for a minimum of 48 hours before further inoculations were made for liquid cultures in TYES. The incubation of bacterial cultures for experimental infection was done according to Madsen and Dalsgaard (1999).

Challenge models

An intraperitoneal (IP) challenge was chosen to investigate the disease resilience to infection with *Flavobacterium psychrophilum* in the three emergence fractions (termed Early, Intermediate and Late) of rainbow trout, when the fish had reached the average weight of 2.3 g. The experimental infections were done as described by Madsen and Dalsgaard (1999). The IP injection method has been shown to be a reproducible infection method resulting in high mortalities when it comes to *F. psychrophilum* in comparison to a bath infection method, where mortalities are lower and the reproducibility not as high (Madsen and Dalsgaard 1999). Before and after challenge the fish were kept in replicated 8 L tanks supplied with water in a flow-

through system (~2 L h^{-1}). The fish were fed dry commercial pellets (Aller Aqua) at 1 % biomass per day and kept at 12 °C. The aquaria and the condition of the fish were monitored at least three times per day, and moribund fish were collected and counted. Moribund fish were killed by an overdose of 3-aminobenzoic acid ethyl ester (MS-222, Sigma A-5040) in water, and samples from inner organs (brain, kidney and spleen) were streaked onto TYES agar and blood agar (BA) plates and incubated at 15 °C for five days up to three to four weeks. Yellow colonies that showed growth on TYES agar but not on BA was identified as *F. psychrophilum* by either a species-specific PCR with DNA primers against a sequence of the *16SrRNA* gene (Wiklund et al., 2000) or by MALDI-TOF. The IP challenge experiment was terminated after 46 days. Thereafter, the surviving fish were killed by an overdose of MS-222, and inner organs were sampled as described above.

For all three emergence fractions a total of three replicates each consisting of 60 fish were used. One group serving as a control where fish were injected with sterile TYES media, whereas the other two replicates served as duplicates, every fish was injected with *F. psychrophilum* in a concentration of 5 x 10^3 CFU (colony forming units)/fish. A total volume of 50 µl was injected into the peritoneal cavity of the fish, regardless treatment type. Prior to injection all fish were anaesthetized with MS-222.

Analysis of plasma stress markers

Plasma cortisol concentrations were measured using a commercial ELISA kit (# 402710 Neogen Europe, Ayrshire, Scotland, UK), following the manufacturer's instructions. The kit showed good linearity, parallelism and reproducibility (intra-assay and inter-assay coefficients of variation lower than 2 % and 5 %, respectively). Plasma glucose and lactate were analyzed with colorimetric kits from Sigma (St. Louis, MO, USA).

Analysis of forebrain serotonergic activity

The brain of each individual was dissected out from the frozen head and immediately processed for the analysis of serotonergic activity. The forebrain, including olfactory bulb, telencephalon, optic tectum and hypothalamus, was homogenized in 0.3 mL of a 4 % perchloric acid solution. After centrifugation of the homogenate, a diluted aliquot of the supernatant was analyzed using high performance liquid chromatography with electrochemical detection (HPLC-EC) as previously described (Gesto et al., 2017). The levels of serotonin (5-HT) and its main oxidative metabolite 5-hydroxyindoleacetic acid (5-HIAA) were quantified by comparing peak areas with those of corresponding standards. The ratio between 5-HIAA and 5-HT was then calculated as an indirect measure of the activity of serotonergic neurons (Winberg and Nilsson, 1993).

Statistics

Fish mass data were analyzed by two-way ANOVA, using emergence fraction and repeated stress as variables. Plasma stress markers and brain serotonergic activity were analyzed by three-way ANOVA, with emergence fraction, repeated stress and time after acute stress as variables. In every case, Holm-Sidak post-hoc tests followed the ANOVA, to detect differences among groups. In all cases, differences were considered significant at $P \leq 0.05$. SigmaPlot version 12.5 (Systat Software Inc., San Jose, CA, USA) was used for these statistical analyses.

Survival data post-selection and survival data from the IP challenge experiment was fitted a Cox proportional hazards model (Therneau & Grambsch 2000) using R (R Development Core Team 2011) which takes replicate aquaria into consideration. Prior to fitting, the assumption of proportional hazards was evaluated (Grambsch & Therneau 1994). Statistical significance ($p < 0.05$) of survival between fractions were tested with a Wald test, which does not assume independence of observations within the replicates.

RESULTS

Initial mortality of larvae

The survival of the larvae during the first 40 days after selection was different for each of the three emergence fractions (Figure 1). With the Early fraction as reference both the Intermediate and Late fraction showed significantly lower survival ($P = 5.82 \times 10^{-6}$ and $P = 6.01 \times 10^{-12}$, respectively). Furthermore, the survival of the Late fraction was also significantly lower than the Intermediate fraction ($P = 5.77 \times 10^{-6}$). The natural mortality decreased with time and declined in all fractions 20 days after leaving the sorting devices (Figure 1).

Fish mass

The repeated stress protocol had an effect on fish final mass ($P < 0.001$), which was independent of the emergence fraction of the fish ($P = 0.484$). The mass of repeatedly-stressed fish from the Intermediate and Late fraction was significantly decreased with respect to the corresponding control fish (Figure 2).

Stress resilience experiment

Plasma cortisol

There was a general effect of emergence fraction and the Early and the Late fractions showed higher cortisol levels than the Intermediate fraction. There was also a general effect of the time post-stress (TPS): cortisol levels were higher at 1 h than at any other time. The temporal response of cortisol to the acute crowding stressor depended on the previous repeated stress protocol and on the emergence fraction of the fish (Figure 3). The cortisol levels of naïve fish from all fractions increased 1 h after stress start and were again at control levels at 2 h. This cortisol increase was higher in the Early than in the Intermediate and late fractions. The repeated stress protocol modified this response in a different manner depending of the emergence fraction of the fish. In the Early fraction, the cortisol response was abolished in the repeatedly-stressed fish. In the Intermediate fraction, the cortisol response was delayed in the repeatedly-stressed fish, peaking at 2 h and only recovering at 4 h post-stress. In the late fraction, the cortisol response of the fish exposed to the repeated stress protocol was atypical, and fish showed high cortisol levels before the acute crowding stress, which decreased at 2 h after stress.

Plasma glucose and lactate

Minor differences were observed in the response of glucose to stress (Table 1). In general, glucose levels were higher at 1 h and 2 h after stress than at 8 h, independently of emergence fraction or previous exposure to the repeated stress protocol. In general, glucose levels were higher in the Late than in the Intermediate fraction, regardless time post-stress and the previous exposure to the repeated stress protocol. There was also an interactive effect of fraction and repeated stress on glucose levels, which were higher in the Late than in the Early fraction in naïve fish, and were lower in the Intermediate than in the Early and Late fractions in the fish exposed to the repeated stress protocol. In fish from the Early fraction, glucose levels were higher in fish exposed to the repeated stress protocol.

All three factors tested (emergence fraction, repeated stress and time post-acute stress) had an effect on plasma lactate levels, but no interactions among their effects were detected. Lactate increased at 1 h post-stress, and then decreased to an intermediate level, independently of emergence fraction or previous exposure to the repeated stress protocol (Table 1). Also, lactate was in general higher in fish from the Late than the Early or Intermediate fractions. Finally, lactate was also higher in naïve fish when compared to fish exposed to the repeated stress protocol.

Brain serotonergic activity

Fish exposed to the repeated stress protocol showed higher levels of 5-HIAA, 5-HT and 5-HIAA/5-HT ratio in the forebrain, regardless time post-stress or emergence fraction. Data showed also a general effect of time post-stress on the serotonergic ratio, which showed increased values at 1 h post-stress, and then returned to control levels, independently of emergence fraction or previous exposure of the fish to the repeated stress protocol (Table 2). Those changes in the ratio were mediated by changes in the levels of 5-HIAA, which showed similar results, since alterations in the levels of 5-HT were minor in this study. An effect of emergence fraction, independent of repeated stress or time post-acute stress was found for serotonin: fish from the Early fraction showed a higher concentration of 5-HT than those of the Late fraction.

Flavobacterium psychrophilum challenge

There were no significant differences in survival between the fish in the Early, Intermediate and Late fractions ($P > 0.05$) (Figure 4). Median survival times for the duplicate aquaria in the Early fraction were: 24.5 and 27 days; the Intermediate fraction: 31 and 23 days and the Late fraction: 21 and 25.5 days. In all three fractions mortality started to occur at day seven and declined around day 35. The remaining fish were then kept under observation for an additional week, to make sure no further mortality would occur. Final mortality for the duplicate aquaria in the Early fraction was: 62 and 62 %; the Intermediate fraction: 56 and 59 % and the Late fraction: 68 and 58 %. *Flavobacterium psychrophilum* were isolated from at least one of the three organs tested (brain, kidney and spleen) in all moribund fish. There were three control aquaria in the IP challenge model where the fish were only injected with sterile TYES media. No mortality was observed in the Early and Intermediate control fractions, however, one out of 30 fish died in the Late control fraction during the experimental trial of 46 days. This casualty was not associated with the presence of *F. psychrophilum* in any of the tested organs.

DISCUSSION

Just after the fish larvae were selected according to their emergence time, they were allocated in new tanks and were given feed for the first time. During the first 20 days of the weaning stage, the mortality was higher in the fish that needed more time to develop the swim-up behavior. The reason behind this difference in larvae mortality in this study is not known. It is possible that a higher mortality in the later fractions can be related to

a higher prevalence of developmental problems affecting the digestive system, preventing fish to be able to catch, swallow or digest feed pellets, when their yolk was consumed, or the locomotion system, preventing fish to be able to properly swim and reducing their possibilities to reach the exit of the artificial nest used in this study at the proper time. A delayed time for first feeding has been shown to affect larval mortality in fish since it can induce a progressive deterioration of the larval digestive system and atrophy of skeletal muscle fibres (Gisbert et al., 2004).

When naïve fish from the different fractions were exposed to the acute stressor, their cortisol response was similar regarding its dynamics, but was different in terms of amplitude. As expected, all fractions showed an activation of the HPI axis at 60 min after the acute stress challenge, resulting in higher levels of cortisol in the plasma. In all fractions, the cortisol levels were again at pre-stress levels two hours post-stress but in the Intermediate and Late fractions the cortisol levels increased at 4 h to levels which were not statistically different than those at 60 min post-stress. However, the intensity of the HPI activation was higher for the Early fraction, which showed cortisol levels around two times higher than those of the Intermediate and Late fractions. A higher cortisol response can be seen as an indicator of a higher level of stress, since the cortisol response is known to be gradable according the subjective severity of the stressor (Gesto et al., 2013). Paradoxically, cortisol levels can also integrate information about the stress experienced by the fish during their lifetime, and lower levels of cortisol upon exposure to an acute stress challenge could be also indicative of chronic, prolonged or repeated exposure to stress (Culbert and Gilmour, 2016; Jeffrey et al., 2014). For example, studies in rainbow trout and sea bream have shown lower HPI reactivity to acute stress in fish that had been held at high stocking density (Barton et al., 2005; McKenzie et al., 2012; Moltesen et al., 2016; Vijayan and Leatherland, 1990) or in Atlantic salmon exposed to repeated unpredictable stress (Madaro et al., 2015). For example, Barton and collaborators (2005) showed that the cortisol response of gilthead sea bream to an acute stressor was 50 % smaller in fish that were reared at high density, compared to fish reared at low density. Madaro and collaborators (2015) showed also a down-regulation of the cortisol response to a novel stressor after submitting salmon parr to repeated unpredictable chronic stress for 23 days. Similarly, chronic social stress has been shown to down-regulate HPI reactivity in rainbow trout or Arctic charr (Gilmour et al., 2005; Øverli et al., 1999; Sloman et al., 2002). This suppression of the HPI reactivity has been suggested to be to be related with a state of increased allostatic load (Madaro et al., 2015; Moltesen et al., 2016) or to a prolonged cortisol-mediated down-regulation of the HPI axis that can even lead to an exhaustion of the HPI axis (Barton et al., 1987; Basu et al., 2002; Hontela, 1997; Marentette et al., 2013; Vijayan and Leatherland,

1990). In the current study, the intense cortisol response and the fast and robust recovery in the Early fraction point to a better performance of the HPI axis in this group, which may be indicative of a higher resilience against stress.

The effect of the repeated stress protocol on the acute stress response was dependent on the emergence fraction of the fish. In the Early fraction, the repeated stress protocol induced a lack of response of the HPI axis to the acute stress protocol. It has been shown that fish, as other vertebrates, can habituate to mild, repeated and predictable stressors (Fast et al., 2008; Jentoft et al., 2005; Madaro et al., 2016) and therefore, a process of habituation could explain the lack of HPI reactivity to the acute stress challenge in the Early fraction. The stress protocol used in the present study to induce the acute stress response was not exactly the same that the stressor used for the repeated stress exposure, however, in a way, they were very similar. In this regard, the stressor utilized for the acute stress challenge (high stocking density) was initiated in the same way than the stressor used for the repeated stress (air exposure). In both cases, the aquaria containing the fish were lifted from the bottom of the tanks. In the case of the acute stressor, the aquaria were not lifted enough to expose the fish to the air. Similarly to the lack of response of the HPI axis observed here for fish of the Early fraction submitted to repeated stress, Madaro and collaborators (2016) showed a strongly reduced cortisol response to acute handling stress in Atlantic salmon after repeated exposure for two or more days. In the same species, fish showed signs of habituation to a daily episode of crowding at day 64 but not at day 29 (Basrur et al., 2010), which highlights the influence of stressor type and stressor severity on habituation ability. On the other hand, the fish from the Intermediate and the Late fractions showed a different response to the repeated stressor. In the case of the Intermediate fraction, the repeated stress altered the normal acute stress response, delaying the cortisol peak and the recovery of basal cortisol levels. In the Late fraction, the cortisol levels in the fish exposed to the repeated stress did not suggest a habituation to the stressor, either. Cortisol levels were higher than in the other fractions at time zero, even before applying the acute stress challenge, maybe indicating an anticipatory response to the stressor or a lack of recovery from the previous stress episode the day before.

The lower HPI reactivity after the repeated stress, together with a better ability to keep a similar growth rate than naïve fish (reflected in the lack of effects of the repeated stress on fish final mass), point to a better stress-habituation capacity of the fish from the Early fraction, suggesting that the Early fraction was the most stress-resilient. Therefore, the reduced cortisol response to the acute stress protocol in the naïve fish from the Intermediate and Late fractions could be indicative of repeated or prolonged activation of the HPI axis in those fish, as commented before. The apparent differences in the ability to habituate to the predictable stressor

among the different fractions may rely on differences in the subjective severity of the stressor. The habituation to repeated stressors in vertebrates is believed to be faster and more complete when the stressor is less severe (Grissom and Bhatnagar, 2009; Herman, 2013) and our present results seem to be in line with that assertion: The Early emergence fraction, showing a more robust response to a novel stressor (probably indicating a subjective perception of the stressor as of lower severity), is also the fraction showing a better habituation to it upon repeated exposure.

In spite of the differences found in the cortisol response, little differences were found among fractions in the other stress markers used in this study. Glucose levels are known to increase after exposure to different stressors in fish, due to the actions of both catecholamines and cortisol on the liver intermediary metabolism (Mommsen et al., 1999; Wendelaar Bonga, 1997). In this study, the profiles of the glucose response were similar to those of cortisol, but in general reached no statistical significance. Interestingly, there were some general emergence time related differences in plasma glucose levels, which were affected by the previous exposure to repeated stress. The reason behind those differences is not obvious since glucose levels are known to vary depending on many factors in fish outside stress responses, including feed intake, basal metabolism, previous events experienced by the fish, etc. (Oliveira et al., 2013; Sopinka et al., 2016). In any case, both glucocorticoids and catecholamines are known to have key roles in the control of glycaemia (Fabbri and Moon, 2016; Mommsen et al., 1999) and therefore, the observed differences in glucose levels could be in part mediated by the adaptation to different conditions of activation of the BSC and HPI axes during the fish lifetime.

Muscle lactate levels are also known to increase in fish after exposure to different stressors as a result of both an increase in muscle activity and a shortage in the O_2 delivered to the muscle, leading to increased rates of glycogenolysis in anaerobic conditions (Milligan and Girard, 1993; Van Ham et al., 2003). The amount of muscle lactate released to circulation is species-dependent in fish and has been reported to be around 10-20 % of the total lactate produced in salmonids (Milligan and Girard, 1993). In this study, there was a clear increase of plasma lactate levels after the acute stress challenge, but the emergence fraction or the repeated stress protocol showed no interactions with the acute stress effect. Interestingly, the repeated stress protocol induced a decrease of plasma lactate regardless emergence fraction or time post-acute stress. Once present in fish tissues, lactate can be oxidized or be incorporated again into glycogen, and it is possible that the fish exposed to the repeated stress have adapted to episodes of increased lactate (as a result of the hypoxia induced by the air exposure), becoming more efficient in metabolizing it.

Very interestingly, there were no differences among emergence fractions in the brain serotonergic activity of the fish, in spite of the different HPI reactivity. In fish, as in other vertebrates, an increase of the activity of serotonergic neurons in certain parts of the brain is one of the primary elements of the response to acute stress (Gesto et al., 2013). The serotonergic activation has been suggested to be part of the mechanisms participating in stress recognition and in the organization of the integrated stress response (Gesto et al., 2013; Winberg and Nilsson, 1993). The ratio between 5-HIAA and serotonin is often used as an indirect estimator of the serotonergic activity (Winberg and Nilsson, 1993) and has been shown to increase after exposure to different types of stress in fish (Conde-Sieira et al., 2014; Gesto et al., 2013; 2015; 2016; Øverli et al., 2001; Winberg and Nilsson, 1993). In this study, a general increase in serotonergic activity was found at 60 min post-stress and, as use to happen after stress, the changes in the serotonergic ratio were mostly based on changes of the metabolite 5-HIAA rather than on the serotonergic levels, which remained mostly unaltered. However, no effects of emergence fraction or the exposure to repeated stress were found in the serotonergic system. Following previous studies in salmonids, it was suggested to be a correlation between emergence time and SCS, which in turn is believed to be associated with some differences in the serotonergic system between proactive and reactive fish (Øverli et al., 2001; Schjolden et al., 2006; Winberg et al., 2016). For example, Thornqvist and collaborators (2015) found that salmon that emerged later were less bold and showed a higher level of activation of the serotonergic system upon exposure to acute stress than fish that emerged earlier, this data suggesting an association between emergence time, boldness and some traits of the serotonergic response to stress. The results of the current study do not appear to support a similar link between emergence time and the response of the serotonergic system to stress. The only difference found among fractions in the serotonergic system was a lower overall serotonin level in the forebrain of the Late versus the Early fraction. However, the response of the serotonin system to the stress protocols was very similar for all the fractions and in this regard, our data do not support the view of the different emergence fractions as fish of different SCS. The lack of consistency in this regard compared to previous salmonid studies could reside in the genetic background and the level of domestication of the fish utilized in the experiment, as discussed elsewhere (Gesto et al., 2017).

Interestingly, the habituation to stress seen in the cortisol levels in fish of the Early fraction exposed to the repeated stress protocol was not reflected at the level of the brain. Fish from all the fractions, exposed or not to the repeated stress, responded to the acute challenge with a similar activation of the brain serotonergic system. Therefore, the lack of HPI reactivity in the repeatedly-stressed Early fraction was not the result of a

reduced activation of the serotonergic system. This suggest that the mechanism in charge of reducing the HPI reactivity during stress habituation must reside somewhere else, but currently, little is known about stress habituation in fish and the potential mechanisms involved.

In addition to the responses of the fish toward stressors, it is highly important to evaluate the resilience of the fish towards diseases. Disease outbreaks with the bacterium *Flavobacterium psychrophilum* cause high losses in salmonid aquaculture worldwide (Nematollahi et al., 2003). Fish infected with *F. psychrophilum* have high mortality rates, and fry are especially affected with mortalities up to 80-90 % (Jensen et al., 2003) if left untreated. Methods of sorting early hatched fry to retain disease resilient fish, e.g. based on principles such as emergence time, are therefore of high value. However, in the present study the tested challenge did not show any significant difference in survival among the three fractions Early, Intermediate and Late. The IP model has demonstrated replicable results and intermediate mortalities, which are highly usable for interpretation between groups of fish (Madsen and Dalsgaard, 1999). The mortality rate and final survival of the fish in the IP challenge model were equal to previous studies in our lab on control rainbow trout of the same size (e.g., Madsen and Dalsgaard, 1999, 2000). The life history, including the sorting process, of the fish used in the present study do therefore not seem to have negatively affected the disease resilience of the fish towards infection with *F. psychrophilum*.

The results of the present study demonstrated that fish selected according to their emergence time were different in terms of their neuroendocrine response to an acute stressor, as well as in the way those neuroendocrine responses were modified upon exposure to a repeated stressor. The different emergence fractions showed clear differences in the HPI axis activation upon exposure to different stress events. However, this fact seemed to have no relevance in the ability of the fish to perform well. The fish from the different fractions were not different in terms of growth (either before, or during the experiment) or, as demonstrated here, in their ability to overcome an infection challenge with a common salmonid disease. Actually, fish from the same emergence fractions used in this study were also tested, several months later, for their stress response and their ability to compete for food and the results showed no differences in their performance (Gesto et al., 2017). Therefore, all this suggests that, at least in an environment relatively free of intense stressors, the neuroendocrine differences in the stress response had little relevance in the ability of the fish to perform well, and this indicates that selecting fish according their emergence time would probably be of little use to improve the welfare of farmed rainbow trout.

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452 **COMPETING INTERESTS**

453 No competing interests declared

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Table 1. Plasma glucose and lactate after an acute stress challenge in fish of different emergence time (Early, Intermediate, Late), previously exposed ("Repeated stress", RS) or not ("Control") to a daily stress protocol for 15 days (see the text for details).

A

		Time post-acute stress				
Glucose (mM)		0 h	1 h	2 h	4 h	8 h
	Early Control	1.97 ± 0.18	2.44 ± 0.17	2.19 ± 0.16	2.11 ± 0.12	1.79 ± 0.19
	Early RS	2.60 ± 0.19	2.71 ± 0.22	2.57 ± 0.21	2.35 ± 0.08	2.42 ± 0.15
	Intermediate Control	2.00 ± 0.15	2.48 ± 0.17	2.36 ± 0.18	2.32 ± 0.13	2.14 ± 0.19
	Intermediate RS	1.92 ± 0.15	2.09 ± 0.14	2.44 ± 0.20	1.88 ± 0.13	2.07 ± 0.09
	Late Control	2.33 ± 0.18	2.54 ± 0.15	2.47 ± 0.22	2.39 ± 0.16	2.14 ± 0.17
	Late RS	2.45 ± 0.12	2.47 ± 0.17	2.37 ± 0.20	2.40 ± 0.19	2.04 ± 0.10
Lactate (mM)						
	Early Control	0.77 ± 0.06 a	1.73 ± 0.20 b	1.24 ± 0.12 ab	1.13 ± 0.10 ab	1.02 ± 0.11 a
	Early RS	0.78 ± 0.07 a	1.39 ± 0.14 b	1.17 ± 0.15 ab	0.91 ± 0.06 ab	1.22 ± 0.15 ab
	Intermediate Control	1.10 ± 0.09	1.60 ± 0.12	1.23 ± 0.25	1.30 ± 0.13	1.18 ± 0.11
	Intermediate RS	0.90 ± 0.08 ab	1.38 ± 0.24 a	1.36 ± 0.15 a	0.95 ± 0.32 b	0.90 ± 0.07 ab
	Late Control	1.13 ± 0.08	1.68 ± 0.21	1.32 ± 0.11	1.34 ± 0.10	1.34 ± 0.10
	Late RS	0.93 ± 0.15 a	1.46 ± 0.18 b	1.28 ± 0.15 ab	1.33 ± 0.16 ab	1.20 ± 0.06 ab

B

		Fraction			Repeated stress		Time post-acute stress				
Glucose	P-value	Early	Intermediate	Late	Yes	No	0 h	1 h	2 h	4 h	8 h
Factors											
Fraction (F)	0.046	ab	a	**b**							
Repeated stress (RS)	ns										
Time post-acute stress (TPS)	0.002						AB	**A**	**A**	AB	B
Interactions											
F x RS	< 0.001	*RS: yes > no			*F: Early > Intermediate Late > Intermediate	*F: Late > Early					
F x TPS	ns										
RS x TPS	ns										
F x RS x TPS	ns										
Lactate											
Factors											
Fraction (F)	0.012	a	a	**b**							
Repeated stress (RS)	0.002				x	**y**					
Time post-acute stress (TPS)	< 0.001						A	**B**	C	C	C
Interactions											
F x RS	ns										
F x TPS	ns										
RS x TPS	ns										
F x RS x TPS	ns										

Data are mean and s.e.m. of n = 10 - 13 for a given emergence fraction, repeated stress treatment, and time post-acute stress. In panel A, different letters indicate significant differences among time points for a given emergence fraction and repeated stress treatment. In panel B, different letters indicate significant differences

among the different categories of a given factor (the bold letter indicates the category showing the higher values of a given parameter (Three-way ANOVA, $P \leq 0.05$).

Table 2. Forebrain levels of serotonin (5-HT), its metabolite 5-hydroxyindoleacetic acid (5-HIAA) and the % 5-HIAA/5-HT ratio after an acute stress challenge in fish of different emergence time (Early, Intermediate, Late), previously exposed ("Repeated stress", RS) or not ("Control") to a daily stress protocol for 15 days (see the text for details).

A		Time post-acute stress				
5HT (ng g^{-1})		0 h	1 h	2 h	4 h	8 h
	Early Control	553.33 ± 11.96	558.70 ± 22.99	535.95 ± 16.77	556.85 ± 14.84	542.38 ± 21.98
	Early RS	596.67 ± 20.48	594.47 ± 23.76	537.89 ± 19.43	561.22 ± 18.06	529.05 ± 22.38
	Intermediate Control	507.12 ± 16.20	542.20 ± 14.65	530.94 ± 24.20	538.82 ± 13.70	533.12 ± 21.56
	Intermediate RS	543.41 ± 24.15	564.29 ± 23.23	565.24 ± 10.62	537.06 ± 33.25	576.83 ± 20.03
	Late Control	525.08 ± 21.84	561.43 ± 39.44	540.85 ± 23.81	475.37 ± 14.33	499.09 ± 18.47
	Late RS	517.80 ± 22.99	538.33 ± 21.52	574.85 ± 23.73	517.93 ± 26.46	505.10 ± 20.71
5HIAA (ng g^{-1})						
	Early Control	88.02 ± 6.57	100.88 ± 7.01	89.46 ± 6.17	83.82 ± 3.82	84.69 ± 5.94
	Early RS	86.99 ± 7.24 a	114.27 ± 8.94 b	87.73 ± 5.34 a	89.62 ± 3.76 a	92.45 ± 6.70 ab
	Intermediate Control	69.98 ± 3.36	88.21 ± 6.51	82.80 ± 8.76	89.92 ± 5.82	82.71 ± 6.44
	Intermediate RS	80.29 ± 5.40	93.23 ± 5.43	90.73 ± 4.72	91.01 ± 5.64	96.56 ± 2.37
	Late Control	77.35 ± 5.11	94.21 ± 11.67	83.08 ± 7.57	78.31 ± 4.80	85.81 ± 8.03
	Late RS	85.32 ± 7.43	106.43 ± 7.66	96.19 ± 5.35	82.79 ± 5.43	83.16 ± 5.05
% 5HIAA/5HT						
	Early Control	15.98 ± 1.08	17.97 ± 0.57	16.75 ± 1.16	15.09 ± 0.65	15.57 ± 0.80
	Early RS	14.56 ± 1.10 a	19.13 ± 1.13 b	16.25 ± 0.74 ab	16.06 ± 0.69 ab	17.35 ± 0.84 ab
	Intermediate Control	14.30 ± 0.72	16.21 ± 1.14	15.57 ± 1.32	16.73 ± 1.08	15.59 ± 1.20
	Intermediate RS	15.06 ± 1.32	16.47 ± 0.56	16.97 ± 1.05	17.28 ± 1.35	16.68 ± 0.43
	Late Control	14.77 ± 0.89	17.71 ± 0.89	14.36 ± 0.71	16.49 ± 0.98	17.06 ± 1.33
	Late RS	15.73 ± 1.54	18.77 ± 1.23	16.79 ± 0.78	17.59 ± 0.74	16.52 ± 0.86

B			Fraction			Repeated stress		Time post-acute stress				
5HT		P-value	Early	Intermediate	Late	Yes	No	0 h	1 h	2 h	4 h	8 h
Factors												
	Fraction (F)	0.009	**a**	ab	b							
	Repeated stress (RS)	0.034				**x**	y					
	Time post-acute stress (TPS)	ns										
Interactions												
	F x RS	ns										
	F x TPS	ns										
	RS x TPS	ns										
	F x RS x TPS	ns										
5HIAA												
Factors												
	Fraction (F)	ns										
	Repeated stress (RS)	0.008				**x**	y					
	Time post-acute stress (TPS)	< 0.001						A	**B**	A	A	A
Interactions												
	F x RS	ns										
	F x TPS	ns										
	RS x TPS	ns										
	F x RS x TPS	ns										
%5HIAA/5HT												

Factors									
Fraction (F)	ns								
Repeated stress (RS)	0.046	**x**	y						
Time post-acute stress (TPS)	< 0.001			A	**B**	A	AB	AB	
Interactions									
F x RS	ns								
F x TPS	ns								
RS x TPS	ns								
F x RS x TPS	ns								

Data are mean and s.e.m. of n = 10 - 13 for a given emergence fraction, repeated stress treatment, and time post-acute stress. In panel A, different letters indicate significant differences among time points for a given emergence fraction and repeated stress treatment. In panel B, different letters indicate significant differences among the different categories of a given factor (the bold letter indicates the category showing the higher values of a given parameter (Three-way ANOVA, $P \leq 0.05$).

Figure legends

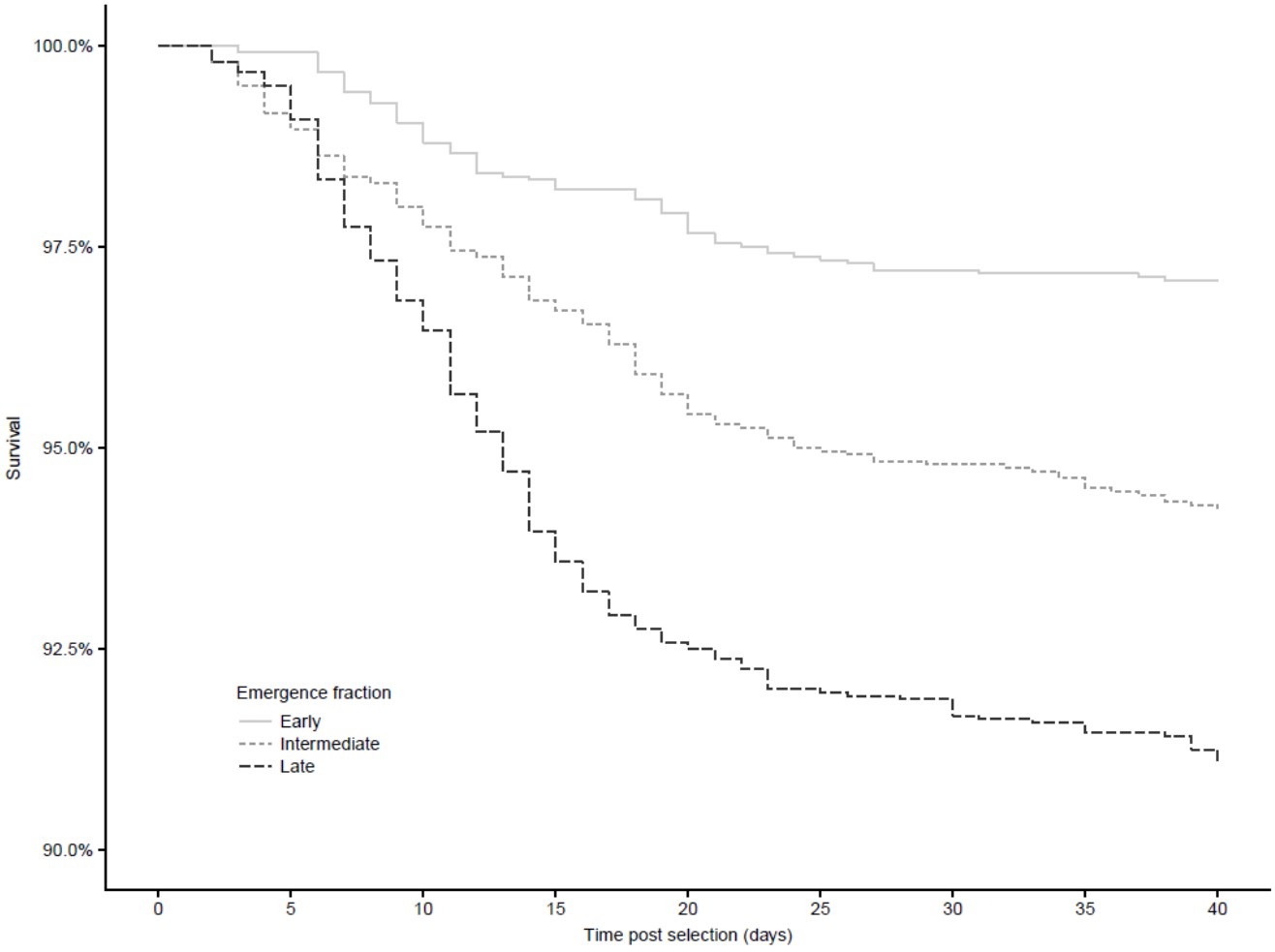
Figure 1. Survival (percent) during the first 40 days after emergence from artificial nests. Survival was higher in the Early fraction than in the Intermediate fraction, which in turn showed higher survival than the Late fraction (Cox Proportional Hazards model, Wald test, $P < 0.05$).

Figure 2. **Average individual mass of fish from different emergence fractions, at the end of the acute stress protocol.** Fish were previously exposed ("Repeated stress") or not ("Control") to a daily stress protocol for 15 days (see the text for details). Data represent the mean and s.e.m. of n = 90 fish per emergence fraction and stress treatment. Asterisks indicate significant effects of the repeated stress protocol within each emergence fraction (two-way ANOVA, $P \leq 0.05$).

Figure 3. **Time course of the response of plasma cortisol to an acute stress challenge in fish from different emergence fractions (Early, Intermediate, Late), previously exposed ("Repeated stress") or not ("Control") to a daily stress protocol for 15 days (see the text for details)**. Data represent the mean and s.e.m. of n = 10 - 13 for a given emergence fraction, repeated stress treatment, and time post-acute stress. Different letters (lower case for "Control" and capital for "Repeated stress") indicate significant differences among time points within each fraction profile. Different symbols (#, ¤) indicate significant differences among fractions within a given time point and stress treatment. The annexed table includes statistical information about the effect of the different factors: Emergence time (EM), Time post-acute stress (TPS) and Repeated stress treatment (RS) (three-way ANOVA, $P \leq 0.05$).

Figure 4. Survival (percent) of fish intraperitoneally challenged with *Flavobacterium psychrophilum*. There were no differences in survival time between the three fractions Early, Intermediate or Late (Cox Proportional Hazards model, Wald test, $P > 0.05$).

642 Figure 1



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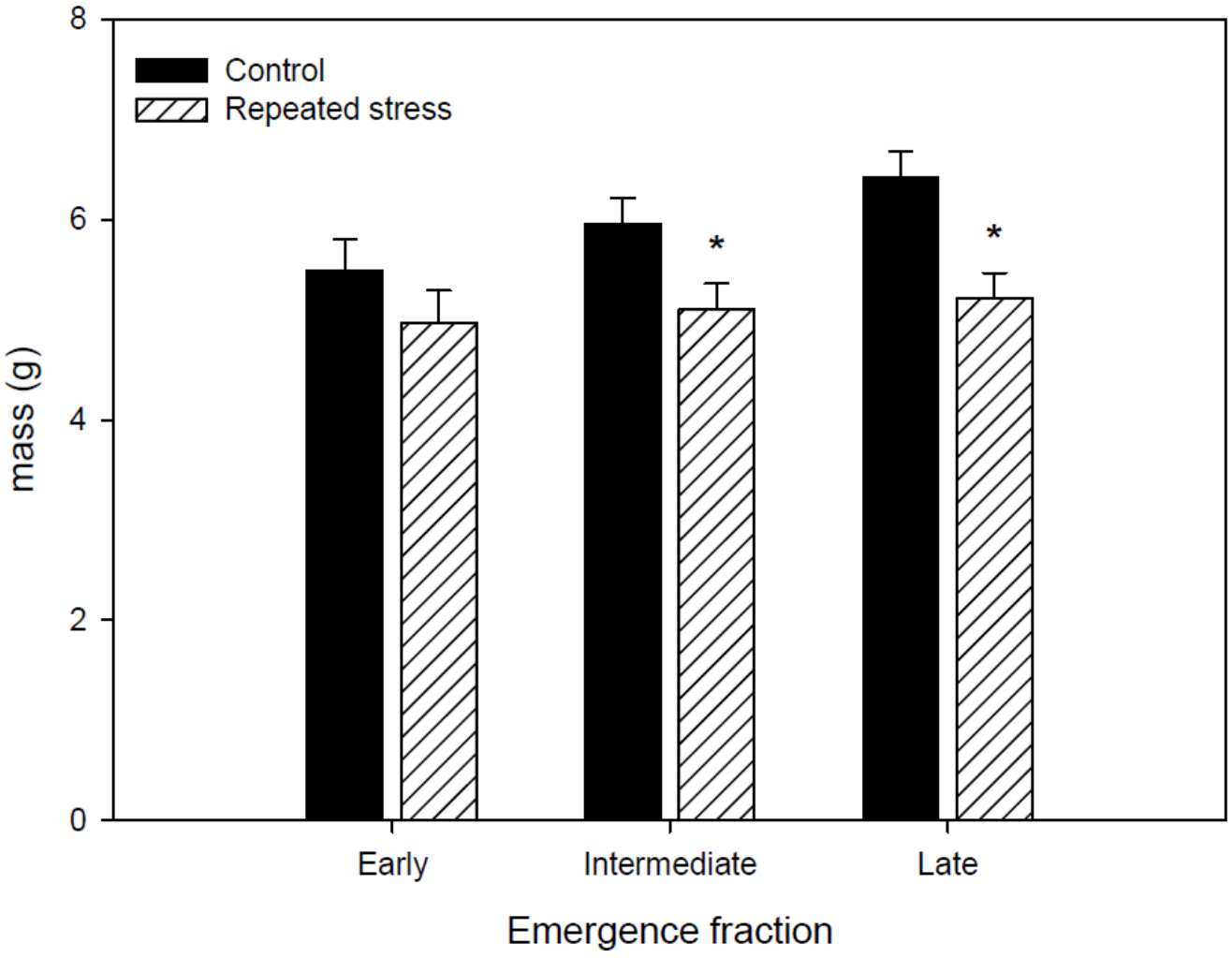
647 Figure 2



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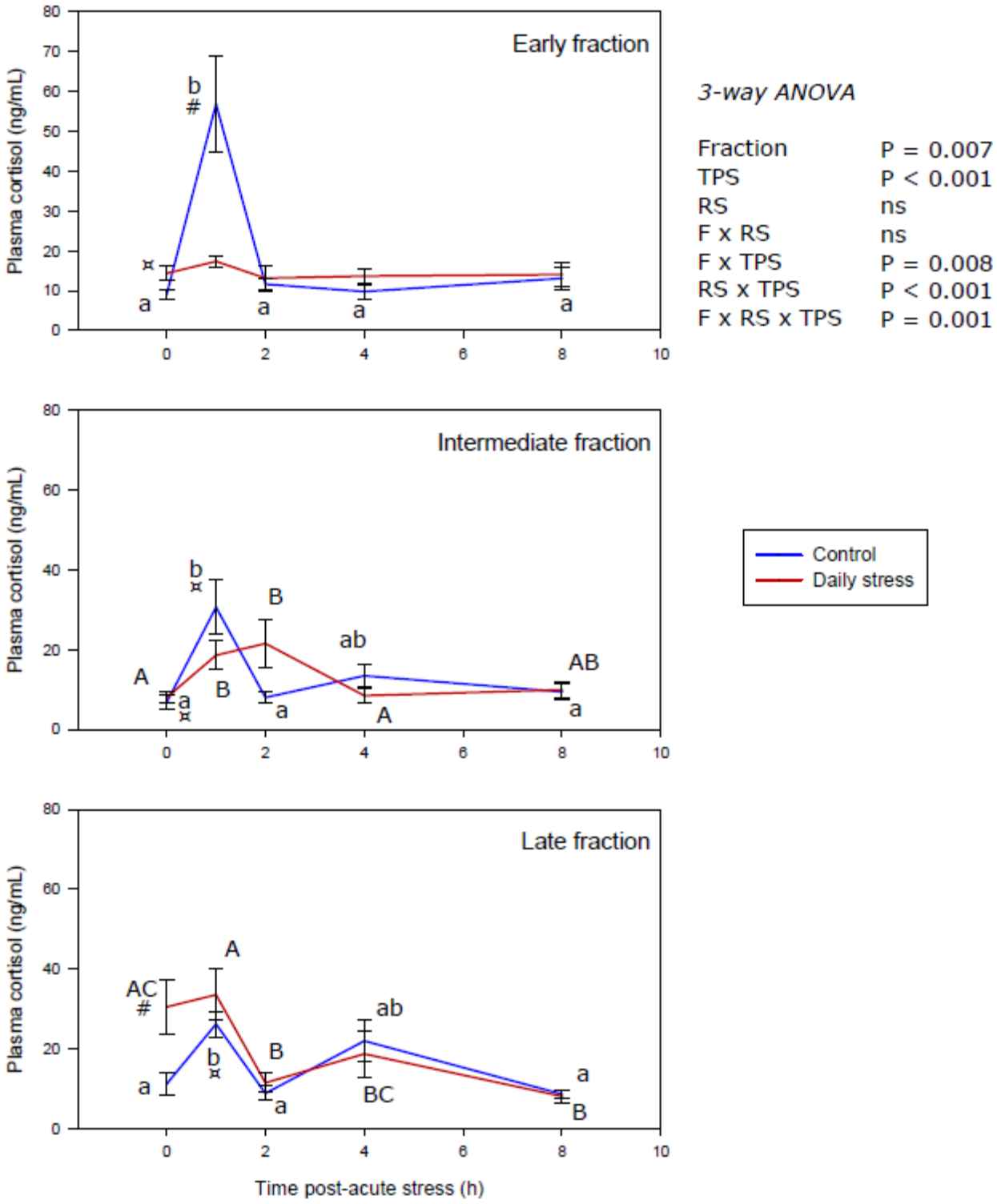
650 Figure 3



3-way ANOVA

Fraction	P = 0.007
TPS	P < 0.001
RS	ns
F x RS	ns
F x TPS	P = 0.008
RS x TPS	P < 0.001
F x RS x TPS	P = 0.001

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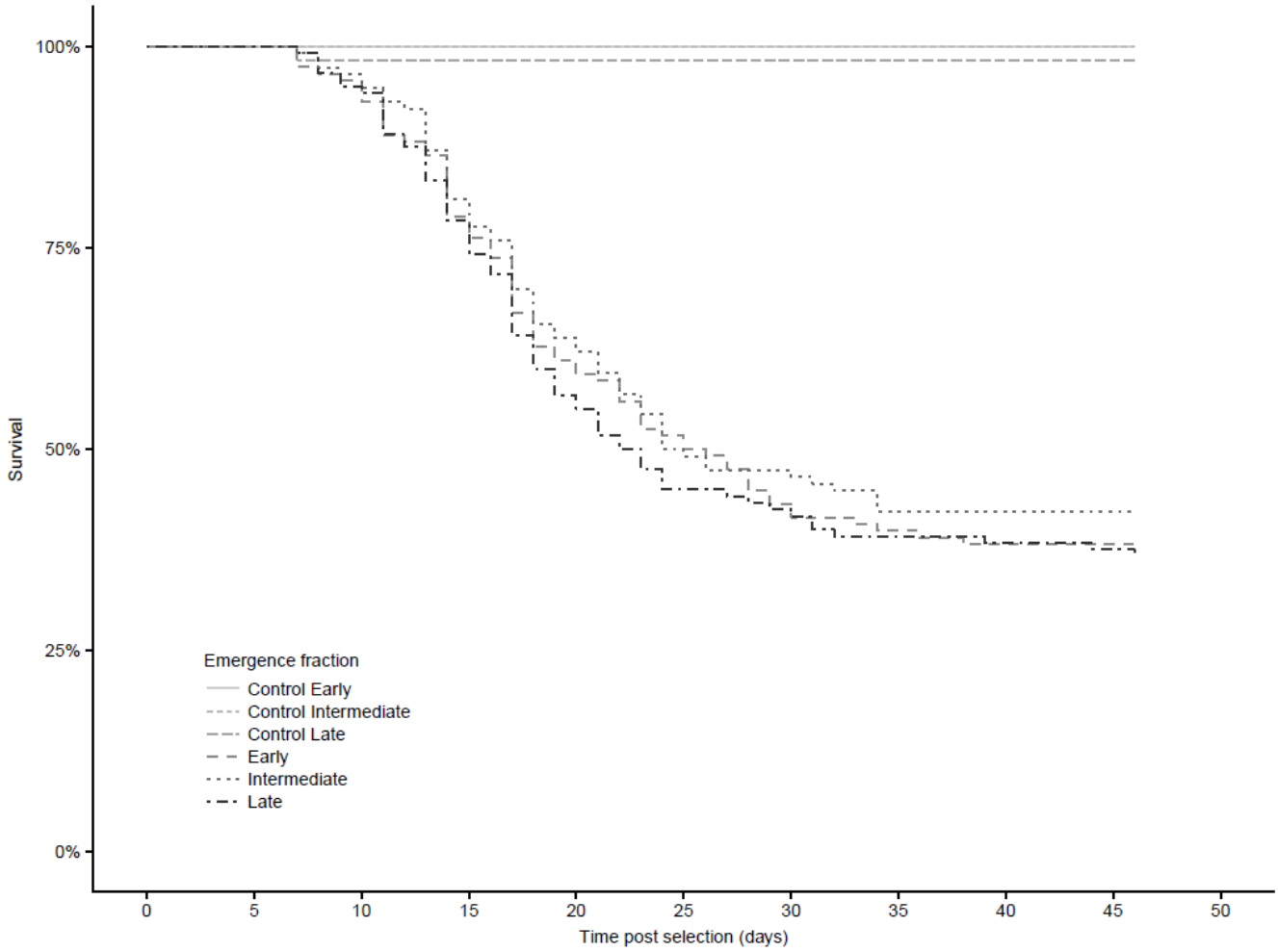
652 Figure 4



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