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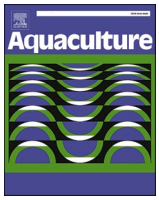

Effects of simple shelters on growth performance and welfare of rainbow trout juveniles

Manuel Gesto *, Alfred Jokumsen

Section for Aquaculture, The North Sea Research Centre, DTU Aqua, Technical University of Denmark, Willemoesvej 2, 9850 Hirtshals, Denmark

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ABSTRACT

Environmental enrichment is a promising strategy to improve the welfare of fish in captivity. However, the utilization of enrichment in aquaculture is still infrequent, maybe because there is a paucity of knowledge about how its effects depend on factors such as fish species, developmental stage, social environment or the type and extent of enrichment. In this study, we evaluated the effects of physical enrichment on the welfare of rainbow trout juveniles, by exposing them to simple plastic screen shelters. Juveniles of approx. 15 g were introduced to two types of submerged shelters: full screens (Full) or partial screens (Semi), and fish welfare was assessed and compared to a control group (without shelters) by evaluating fish growth and condition, extent of external lesions, and the neuroendocrine responses to acute and repeated stress. During the eleven-week experimental period, the fish in the sheltered units gradually developed a clear shelter-seeking behavior when exposed to external disturbance. Fish growth, condition factor and mortality were not affected by shelter presence. The presence of full shelters had a modest protective effect on fin damage: both pectoral fin- and total fin damage scores were reduced (> 10%) in this group with respect to the control group; the percentage of fish with severe damage in the pectoral fin was reduced in the Full group with respect to the Control (63% vs 82%). Partial shelters had no significant effect on fin damage scores, when compared to the control group. The presence of shelters did not affect the general level of stress upon standardized acute or repeated stressors. However, fish used to the presence of shelters showed a more intense startling response when exposed to stressors that forced them to abandon the shelter protection. Altogether, this study shows potential for shelters to be used as a welfare-promoting strategy in trout farming, but further research is needed to optimize the shelter type and design and the proper timing for its application.

1. Introduction

The welfare of fish in aquaculture, public or home aquaria and fish research facilities is of increasing concern (Franks et al., 2021). Hence, there is an urgent need to find solutions, not only to improve, but also to quantify and monitor the welfare of fish in the aquaculture industry (Segner et al., 2019). Threats to fish welfare inherent to fish farming include captivity/confinement itself, inadequate social environments (including stocking density), handling, disease/infection outbreaks and inadequate environmental conditions (water quality, light conditions and others). There is also increasing consensus about the welfare concerns related to the lack of environmental complexity (i.e. lack of variation and/or enrichment) as a result of fish living in barren units (Brydges and Braithwaite, 2009; Sneddon et al., 2016; Xu et al., 2020). It has been shown that animals can benefit from enriched environments and from diversity of external stimulation (Young, 2003). In this regard, the use of different types of environmental enrichment (social, occupational, physical, sensorial or nutritional), has been suggested as a potential strategy to improve the conditions of captive animals from a welfare perspective (Arechavala-Lopez et al., 2021; Näslund and Johnsson, 2016; Young, 2003).

In fish, most studies about the effects of environmental enrichment have focused on physical enrichment, which is based on the deliberate increase of structural complexity by adding different structural elements (most usually floor substrates, shelters, real or artificial plants/algae and toys/novel objects) to the fish rearing units. Reported positive effects of physical enrichment on captive fish include, for example: better survival after release to the wild (Hyvärinen and Rodewald, 2013; Roberts et al., 2014), reduced aggression (Zhang et al., 2020), reduced external injuries (Arechavala-Lopez et al., 2019), reduced stress and startling

* Corresponding author.
E-mail address: mges@aqua.dtu.dk (M. Gesto).

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responses (Rosengren et al., 2017), and positive effects on neural development and cognition (Arechavala-Lopez et al., 2020; Näslund et al., 2012; Salvanes et al., 2013; Zhang et al., 2019). However, observed effects of enrichment are not always positive, and different aspects need to be considered when using environmental enrichment such as type, extent and application timing of the enrichment, species and developmental stage (Ahlbeck Bergendahl et al., 2016; Alnes et al., 2021; Jones et al., 2021; Näslund and Johnsson, 2016; Rosengren et al., 2017; Saraiva et al., 2021; Solås et al., 2019). Furthermore, the introduction of physical enrichment can be also challenging by affecting other aspects such as waste accumulation, routine cleaning procedures, water circulation or even introducing potential pollutants that might leach from the used materials. In this regard, there is a need for further research to fully exploit the potential use of enrichment to promote the welfare of captive and released fish (Arechavala-Lopez et al., 2021).

Shelter-seeking behavior is widely spread among animals to reduce exposure to undesired environmental conditions or to hide when feeling threatened (Aspaas et al., 2016; Haddy et al., 2020; Näslund et al., 2013). Different fish species, including salmonids, have been shown to develop clear shelter-seeking behavior when shelters are available (Champneys et al., 2021; D'Anna et al., 2012; Näslund et al., 2013; Näslund and Johnsson, 2016; Roberts et al., 2011). Most objects or structures deployed as physical enrichment can offer refuge to some extent but very often, the enrichment is purposely designed to provide shelters for the fish. In the case of salmonids, previous studies have shown positive effects of shelter availability on fish physiology (Näslund et al., 2013; Persson and Alanärä, 2014), but inconsistent effects on growth performance were observed (Arechavala-Lopez et al., 2021; Näslund and Johnsson, 2016). Negative effects of shelters on fish growth performance are not necessarily related to poor welfare conditions, but can be the result of a conflict between the motivation for feeding and the motivation for shelter-seeking (Johannesen et al., 2018; Näslund and Johnsson, 2016).

Maybe partly because of that growth performance inconsistency, the use of enrichment in commercial salmonid aquaculture is infrequent, and most of the available studies on enrichment in salmonids are focused on improving the future survival of fish intended to be released for re-stocking purposes. The main aim of the current study was to investigate the potential use of shelters to improve the welfare of rainbow trout in aquaculture. From an operational point of view, any physical structure inside fish rearing tanks can hamper routine tasks in aquaculture farms and therefore, shelter design should consider this. This study thus tested the effects of simple submerged screens on growth performance, external injuries and resilience against acute and repeated stress in rainbow trout juveniles. The stress response of the animals to different stressors was assessed by measuring common blood indicators such as cortisol, glucose and lactate (Wendelaar Bonga, 1997), along with the serotonergic activity in the telencephalon. The brain serotonergic system is believed to have a prominent role in organizing the stress responses in vertebrates (Crawford et al., 2010). Serotonergic activity is consistently increased in fish after stress exposure, particularly in limbic related areas such as the telencephalon (Gesto et al., 2013; Vindas et al., 2017), and it can be viewed as a primary subjective indicator about how the fish perceives a particular stressor.

2. Materials and methods

2.1. Animals and facilities

Rainbow trout juveniles, *Oncorhynchus mykiss* (Walbaum, 1792), weighing 13 g on average, were obtained from a local farm (Lundby Dambrug, Nibe, Denmark) and distributed (200 fish per tank) among nine 600 L-tanks in DTU Aqua's facilities in Hirtshals, Denmark. Fish were left to acclimatize to the rearing facilities for three weeks before starting the trials. During this period, fish were fed at a ration of 1% biomass day^{-1} with commercial pelleted feed (Aller Aqua Futura, 2 mm, Aller Aqua, Denmark). Feeding amounts were corrected on a daily basis based on the predicted growth of the fish (according to own estimates for feed conversion ratio – FCR) and taking into account fish mortality, which was monitored daily. All tanks were part of the same recirculation aquaculture system (RAS), in which the water temperature was kept at 16 °C. Water quality parameters were monitored daily (temperature, pH, oxygen saturation) or every second day (ammonia-N, nitrite-N and nitrate-N) during the duration of the trials. The photoperiod was kept at 14 L:10D (lights on at 7:00). An installed alarm system monitored continuously several key parameters: water level, electricity supply, oxygen saturation level.

2.2. Experimental design and timeline

Three replicate tanks were assigned to each of the experimental conditions: Control (no shelter), Semi (partial screen shelter) or Full (full screen shelter). The experimental groups were assigned in blocks: The nine tanks were arranged formed a line, which was divided into three consecutive subzones, each containing a single tank per condition. The experimental period was started by the introduction of the shelters into the water. At this point, the fish had an average size (SD) of 14.7 g (4.3 g). The shelters were designed as horizontal PVC screens (40 cm × 60 cm, 0.24 m^2), separated 10 cm from the bottom of the tank (Fig. 1). The total area of the tanks was 1 m^2 (1 m × 1 m), so the shelters were covering 24% of the bottom area. The shelters in the Semi group were perforated (7 cm diameter holes; Fig. 1), providing a partial shelter. Feeding rations and water quality monitoring was as described for the acclimation period, until the end of the experiments. Two weeks after the introduction of the shelters, fish numbers were adjusted to 160 per tank. From this moment on, the same setup (i.e. same tanks and same fish) were used for the trials described below and no new fish were stocked into the tanks during the experimental period.

2.2.1. Trial 1. First acute stress challenge

An initial acute chasing stress trial was carried out five weeks after the introduction of the fish to the shelters. The fish were stressed by hitting the water surface repeatedly with a small net and by introducing it in the tanks and moving it around for 1 min without touching the shelters, when present. Net movements were standardized for all tanks. Fish were sampled at 0 min (just before applying the stress protocol), 45 min and 120 min post-stress. At each sampling point, four fish were quickly netted out of the tank and deeply anesthetized in a benzocaine bath (200 mg L^{-1}). The fish were weighed, measured and externally observed for the presence of external lesions in fins, eyes, snout, operculum and skin. A blood sample was then collected from caudal vessels using syringes rinsed with ammonium-heparin solution. Finally, the fish was decapitated and the head immediately frozen on dry ice and later stored at −80 °C. Blood samples were centrifuged (2500 xg, 4 °C, 10 min) and the plasma was stored at −80 °C. External lesions were also evaluated from eight extra individuals from each tank, in order to have 60 individuals assessed in this regard per shelter group. In total, 180 fish (20 per tank, 60 per shelter group) were used in this trial. At the end of the trial, fish numbers were adjusted to 135 fish per tank.

2.2.2. Trial 2. Repeated stress challenge

A second trial was started one week later to evaluate the resilience of the fish when exposed repeatedly to acute stress. Fish were exposed daily to an acute stressor, which was applied at a random time during the light phase of the day. The applied stressor consisted in opening the water outlet of the tank until having a water layer of only 5 cm (50 L approx.). By then, the water outlet was closed and the water levels started to recover at the normal water inflow rate of around 3 L min^{-1}. The stressor was applied daily for seven days and fish were sampled only after the first ("Naïve" fish, exposed only once) and the last ("Repeated stress" fish, stressed daily for seven consecutive days) stress events on day 1 and day 7, respectively. On sampling days, stress and sampling

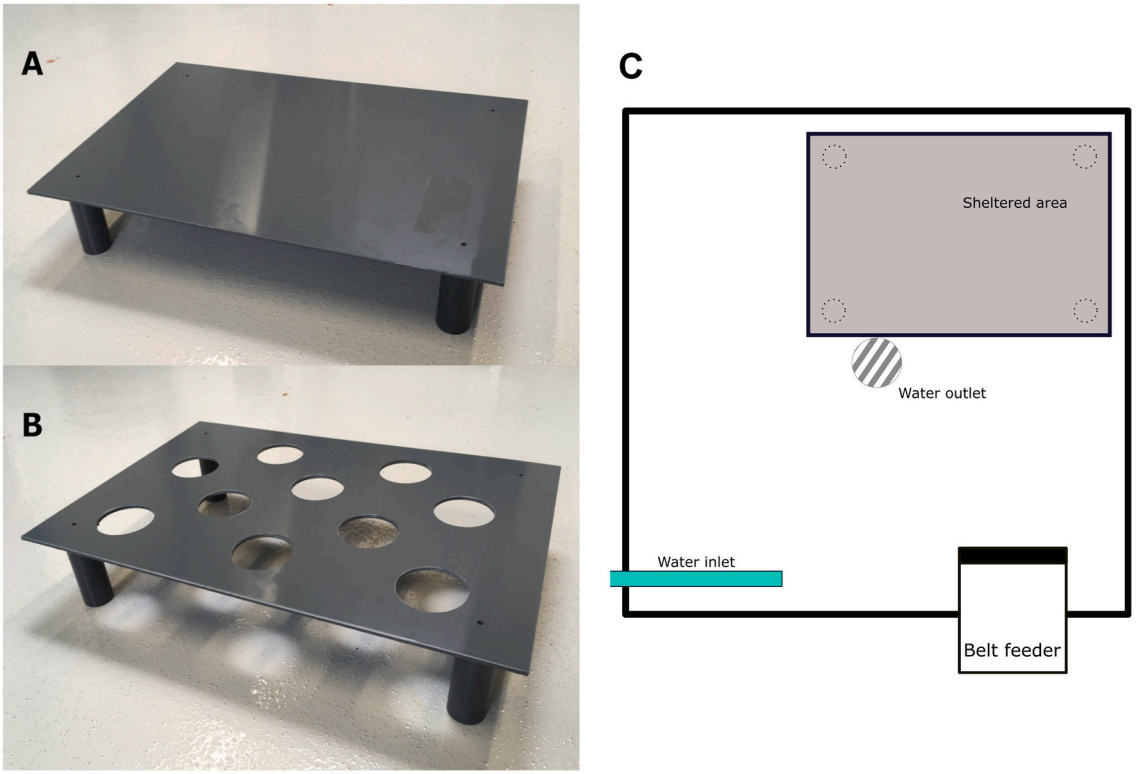

Fig. 1. PVC-screens used in the study as full (Full, A) or partial (Semi, B) shelters. Panel C shows a schematic view (zenithal) of the position of the shelters inside the tanks.

started always at the same time, to avoid the effects of the normal circadian variation in the basal levels of stress markers assessed in the study (Sánchez-Vázquez et al., 2019). Four fish from each tank were sampled 45 min after the water reduction protocol. Sampling was carried out as described for the previous trial. Three additional fish per tank were sampled for external observations (size, fork length and external damage). For logistical reasons, related to the difficulties of applying daily stressors and sampling from nine different tanks at the same time, the experiment was carried out involving only three tanks at a time (one tank per condition). Therefore, three repetitions of the trial (one per week) were carried out during a period of 3 weeks, until involving all tanks. In total, 126 fish (14 per tank, 42 per shelter group) were used in this trial. At the end of the trial, fish numbers were adjusted to 110 fish per tank.

2.2.3. Trial 3. Second acute stress challenge

Another acute-stress challenge was carried out two weeks after the end of the previous experiment, when the fish were already showing clear shelter-seeking behavior (see section on behavior below). The design of this experiment was similar to that of the first trial. In this case however, a combined stress protocol was used with the intention to increase the severity of the stress protocol, which was very mild in the first trial, based on stress marker data. In this case, the stress protocol consisted in reducing the water level of the tanks (as described for trial 2) and chasing the fish with a net for 1 min after that (as described for trial 1). Fish (four per tank) were sampled at 0 min (just before stress), 45 min and 120 min after the acute stress protocol. Sampling was done as described for trial 1. In total, 108 fish (12 per tank, 36 per shelter group) were used in this trial.

At the end of the trials, 11 weeks after the introduction of the shelters, all the fish remaining in the experimental tanks (approx. 100 individuals per tank) were individually weighed, and a subset (40 fish per tank) was measured (fork length) and observed for external damage.

2.3. Assessment of external lesions

External damage at the level of the eye, skin, snout, operculum and fins (pectoral, pelvic, dorsal and caudal) was given a score in a 4-step scale from 0 (undamaged) to 3 (severe damage), following a protocol adapted from Noble et al. (2020). With the exception of the fins, lesions were observed only punctually in very few individuals and therefore, damage to eye, skin, snout or operculum are not reported or discussed in this study. Fin damage scores were assessed for pectoral, pelvic, dorsal and caudal fins. Total fin damage score was also calculated per individual fish as the sum of the scores observed in all 4 individual fins.

2.4. Plasma stress markers

Cortisol was quantified with a commercial ELISA kit (Ref: 402710, Neogen Europe, Ayrshire, UK). Glucose and lactate were analyzed with colorimetric kits from Merck (Darmstadt, Germany; ref.: CBA086) and Megazyme (Bré, Ireland; ref.: K-Late), respectively.

2.5. Brain serotonergic activity

Fish telencephalons were dissected out from the fish frozen heads and immediately processed for the analysis of serotonergic activity. Each individual telencephalon was homogenized by ultrasonic disruption in 400 μL of a 4% perchloric acid solution with 0.1 mmol L^{-1} of ethylenediaminetetraacetic acid (EDTA). After centrifugation of the homogenate, a diluted aliquot of the supernatant was analyzed using high performance liquid chromatography with electrochemical detection (HPLC-EC) as described before (Gesto et al., 2017). Serotonin (5-HT) and its main oxidative metabolite, 5-hydroxyindoleacetic acid (5-HIAA), were quantified, and the ratio between 5-HIAA and 5-HT was calculated as an indirect measure of the activity of serotonergic neurons (Winberg and Nilsson, 1993).

2.6. Shelter seeking behavior

Shelter-seeking behavior was assessed qualitatively, three times during the experimental period: i) one week after the introduction of the shelters; ii) on week 4, three days before trial 1; iii) on week 9, eight days before trial 3. In each occasion, fish shelter-seeking behavior after disturbance (consisting in knocking repeatedly the wall of the tank, followed by waving a small fishing net inside the water) was categorized according to the behavior of the fish immediately after being exposed to the disturbance. The shelter-seeking behavior was categorized in each tank as "absent" (none or few individuals seeking refuge under the shelter), "partial" (presence of relevant fish numbers both under the shelter and in the rest of the tank) or "generalized" (few fish out the sheltered area). Representative videos for each shelter condition were taken underwater with a Hero 7 camera (GoPro Gmbh, Munich, Germany) in all three monitoring events. The camera was introduced in the tanks at least 45 min before exposing the fish to the disturbance. Observations of general fish behavior in relation to the shelter in each tank were made always by the same observer.

2.7. Ethics

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 Experiments Inspectorate (*Dyreforsøgstilsynet*) from the Ministry of Environment and Food of Denmark, under the license number 2019-15-0201-00330.

2.8. Statistical analyses

One way ANOVA was used to compare the effects of the different shelter groups on the initial and final mass, fork length and condition factor K. For the analyses of the fin damage scores, the assumptions for two-way ANOVA analysis were not fulfilled and we followed a two-tier approach at the cost of increased type I error: fin damage scores were compared among the different shelter groups at mid-term (5 weeks) and at the end of the experimental period by Kruskal-Wallis (K—W) tests. Mann-Whitney *U* tests were used to compare the fin damage scores at both sampling times (mid-term vs final), within each treatment group. In all stress experiments, stress-related variables were assessed with the replicate tanks as the experimental units. Data was first averaged by tank ($n = 4$ fish per tank), and then tank averages were used during the statistical analyses ($n = 3$ tanks per shelter treatment). Two-way repeated measures ANOVA were used in the acute stress challenges (trial 1 and 3), with time post stress (0 min, 45 min and 120 min) and shelter group (Control, Semi and Full) as main factors. Two-way repeated measures ANOVA was also used to analyze the data of the repeated stress experiment, using shelter group and repeated stress (Naïve vs Repeated stress) as main factors. Survival data in the different groups was analyzed using Kaplan-Meier curves: Log-rank tests were first used to compared the survival among the three replicated tanks within each treatment. No differences between tanks were found in any of the treatments and, therefore, survival data was grouped per treatment and a long-rank test was used to assess for general survival differences among shelter groups. All statistical analyses were done in Sigmaplot 14.0 (Systat Software, San Jose, CA, USA) and the significant threshold was set at $p \leq 0.05$ in all cases.

3. Results

3.1. Mortality

Survival during the 11-week experimental period (excluding all fish removed from the tanks during the sampling of the different trials) varied between 87% and 90% in the different shelter groups and did not differ significantly between the Full and Semi groups and the Control group ($p = 0.301$ and $p = 0.318$, respectively) (Supplementary Fig. 1).

3.2. Growth performance

The growth of the fish was not affected by any of the shelter types, and the final size and condition factor of the fish was similar for all groups (Table 1).

3.3. Trial 1

Plasma cortisol was affected by time post stress ($p < 0.001$) but not by shelter group or the interaction time x shelter (Fig. 2 and Table 2). Overall, cortisol levels were increased across groups at 45 min after stress, and levels were then recovered at 120 min to levels that were even lower than in control group. Glucose was neither affected by time nor by shelter group. Lactate was not affected by time, but there was a main effect of shelter group ($p = 0.018$): both Semi and Full groups had overall plasma lactate levels that were lower than the in the Control group.

No differences were found in the levels of 5-HT or 5-HIAA across times or shelter groups (Supplementary Fig. 2, Table 2). However, the serotonergic activity was found to be affected by time post-stress ($p = 0.017$), in such a way that the values of the ratio were higher at 45 min than in the 0 min control group (Fig. 2, Table 2). The levels at 120 min post stress were not different to either of the other groups.

3.4. Trial 2

Post-acute stress plasma cortisol and lactate levels were affected by the repeated stress protocol ($p = 0.028$ and $p = 0.024$, respectively), but not by the shelter type or the interaction between both factors (Fig. 3, Table 2). Both cortisol and lactate levels were higher in Naïve fish than in repeatedly exposed fish. Glucose levels were not significantly affected by any of the factors in this trial. The response of brain monoamines to the acute challenge was not affected be either the shelter group or the exposure to repeated stress (Fig. 3, Table 2, Supplementary Fig. 2).

3.5. Trial 3

Plasma cortisol levels were affected by time post stress ($p < 0.001$) but not by shelter type or by the interaction between shelter and time (Fig. 4, Table 2). Overall, plasma cortisol was increased at 45 min post stress and control levels were recovered at 120 min post stress. Plasma lactate levels were affected by shelter type ($p = 0.006$), time post-stress ($p < 0.001$), and by the interaction of both main factors ($p = 0.021$). In the absence of shelters, lactate did not vary after stress exposure. However, lactate was increased at 45 min post stress in both sheltered groups with respect to their respective 0 min group. Furthermore, at 45 min, lactate levels were higher in the Full group than in the Control and Semi groups, while no shelter-related differences were found at 0 min or 120 min. Plasma glucose was not significantly affected by any of the tested factors. At the level of the brain only 5-HIAA and the ratio 5-

Table 1
Initial and final mass, fork length and Fulton's condition factor for the three experimental groups during the 11-week experimental period.

	Initial			Final		
	Mass (g)	FL cm	K	Mass (g)	FL cm	K
Control	14.9 ± 1.1	10.8 ± 0.1	1.14 ± 0.05	52.5 ± 1.8	15.7 ± 0.2	1.35 ± 0.04
Semi	14.4 ± 1.3	10.7 ± 0.3	1.13 ± 0.02	54.6 ± 0.7	15.7 ± 0.1	1.37 ± 0.02
Full	14.9 ± 1.1	10.9 ± 0.1	1.14 ± 0.05	53.3 ± 2.3	15.6 ± 0.4	1.37 ± 0.01

Data is the mean ± SD of n = 60 (Initial) or $n = 280–290$ (Final) fish.

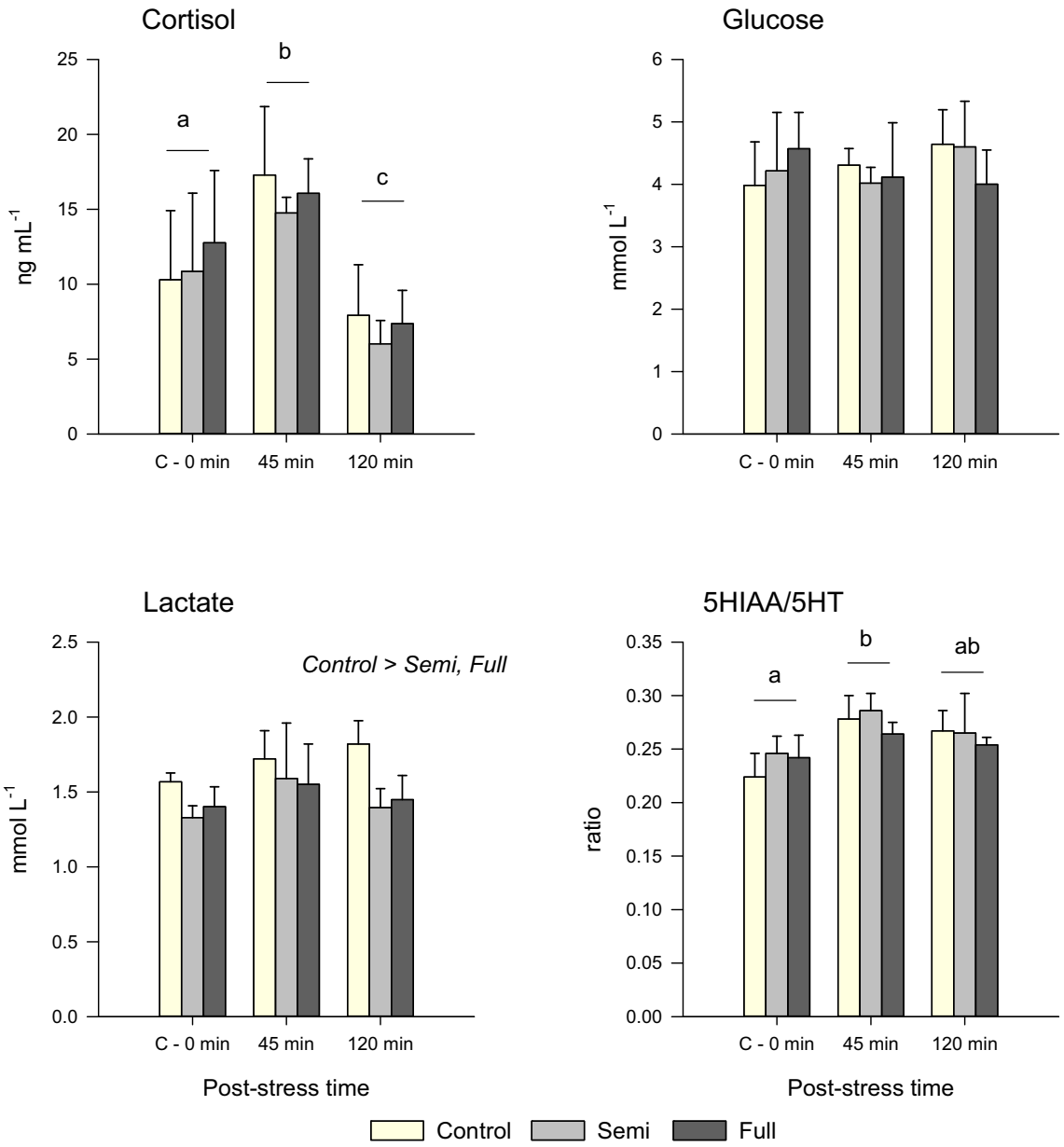

Fig. 2. Cortisol, glucose, lactate and telencephalic serotonergic ratio of the fish in trial 1. Fish were reared for 5 weeks in the presence of partial (Semi) or complete (Full) submerged screen shelters, while Control fish were reared in empty tanks. Fish were then exposed to chasing stress for 1 min and sampled at 0 min (Control), 45 min or 120 min after stress. Data represent the mean and SD of $n = 3$ tanks. Different letters indicate significant differences among sampling times. Shelter-related differences for lactate are indicated in an inset.

HIAA/5-HT were altered in the telencephalon, both affected by the shelter type ($p = 0.009$ and $p = 0.028$, respectively) and by the time post-stress ($p < 0.001$ for both variables), but not by their interaction (Fig. 4, Table 2, Supplementary Fig. 2). Both 5-HIAA levels and the serotonergic ratio increased at 45 min post stress, and then decreased at 120 min to a level that was still above the 0 min group. Overall, the levels of 5-HIAA and the ratio were both higher in the Full shelter group than in the controls.

3.6. Fin damage

Fin damage was registered and compared at two different times during the experimental period: First at 5 weeks (Mid-term) after the start of the experiment, during experiment 1 (20 fish observed per tank, 60 per treatment). Last, at the end (Final) of the second acute stress trial (52 fish per tank, 156 per treatment). The data from both times could not be analyzed together since they were not complying with the required assumptions for multifactorial analysis and therefore, the factor time (mid-term vs final), and the effects of shelters were assessed independently. All individual and total fin damage scores increased at the end of the experiment within each shelter group, with the exception of pectoral and dorsal fins damage scores in the Semi group ($p = 0.304$ and $p = 0.167$, respectively) (Fig. 5, Table 3). No effects of shelters were observed at mid-term, but at the end of the experimental period, both pectoral fin ($p < 0.001$) and total fin damage score ($p < 0.001$) were smaller (11% and 12%, respectively) in the Full than in the Control group. It is important to keep in mind that fin scores were analyzed numerically, since the scores represent a gradient for the extent of fin damage, but they were obtained as a categorical variable. To fully understand the value of fin scores as a welfare indicator, it is also important to know how the fish are distributed among the different damage categories. Therefore, for the sake of completeness, the distribution of the

Table 2
P-values of analyzed stress-related variables of trials 1–3. Significant effects are highlighted in bold.

	Time (t)	t effect	Shelter (s)	s effect	t x s	t x t effect
Trial 1						
Plasma cortisol	< **0.001**	45 min > 0 min > 120 min	0.665		0.687	
Plasma glucose	0.721		0.943		0.607	
Plasma lactate	0.143		**0.018**	Control > Semi, Full	0.718	
Telencephalic 5-HIAA	0.499		0.453		0.964	
Telencephalic 5-HT	0.639		0.924		0.720	
5-HIAA/5-HT ratio	**0.017**	45 min > 0 min	0.108		0.750	
	rep. Stress (rs)	rs effect	shelter (s)	s effect	rs x s	rs x s effect
Trial 2						
Plasma cortisol	**0.028**	Naïve > rep. Stressed	0.406		0.966	
Plasma glucose	0.506		0.661		0.873	
Plasma lactate	**0.024**	Naïve > rep. Stressed	0.845		0.483	
Telencephalic 5-HIAA	0.141		0.232		0.769	
Telencephalic 5-HT	0.669		0.984		0.259	
5-HIAA/5-HT ratio	0.095		0.775		0.382	
	time (t)	t effect	shelter (s)	s effect	t x s	t x t effect
Trial 3						
Plasma cortisol	< **0.001**	45 min > 0 min, 120 min	0.234		0.570	
Plasma glucose	0.736		0.443		0.600	
Plasma lactate	< **0.001**		**0.006**		**0.021**	Semi: 45 min > 0 min Full: 45 min > 0 min, 120 min 45 min: Full > Semi, Control
Telencephalic 5-HIAA	< **0.001**	45 min > 120 min > 0 min	**0.009**	Full > Control	0.142	
Telencephalic 5-HT	0.918		0.776		0.343	
5-HIAA/5-HT ratio	< **0.001**	45 min > 120 min > 0 min	**0.028**	Full > Control	0.188	

fish among the different score categories for each fin is shown in Fig. 6. In general, the calculated average scores were in line with the observed distribution of the fish among the damage categories. The percentage of fish showing a score of 3 (severe damage) in the pectoral fins was 82% in the control group, and was reduced to 63% of the fish in the Full group (Fig. 6).

3.7. Shelter-seeking behavior during the experimental period

Shelter-seeking behavior developed sequentially during the experimental period. One week after introducing the shelters, fish showed no apparent shelter-seeking behavior upon exposure to external disturbance (knocking on shelter walls and waving a net inside the tank). Fish were swimming around the shelter and seemed to actively avoid getting under it. Shelter-seeking behavior was categorized as "absent" in all six sheltered tanks. In the second behavioral test in week 4, part of the fish inside the tank were observed to seek for shelter when the same external disturbance was applied. Many fish in the tanks remained swimming out of the sheltered area. The shelter-seeking behavior was categorized as "partial" in five out of the six sheltered tanks (in one tank with Full shelters, the assigned category was "absent"). Finally, when the disturbance was applied in the third behavior test on week 9, fish in both Semi and Full groups showed a clear shelter-seeking pattern (an illustrative example can be seen in Suppl. video 1). At this time, all/almost all the fish present in the tanks tried clearly to get under the shelters when disturbed, and the shelter-seeking behavior was categorized accordingly as "generalized" in all six tanks. The shelter-seeking behavior was marked even in response to the introduction of the camera in the tank (before applying the standardized disturbance), something that was not observed in the previous tests.

4. Discussion

At the end of the experimental period, the fish had developed a clear shelter-seeking behavior when exposed to an external disturbance (knocking on the wall of the tank followed by surface splashing with a net), in such a way that they accepted to be highly crowded under the shelter to try to get away from the threat. The qualitative assessment of the shelter-seeking behavior did not allow to detect potential differences between both types of shelters, but the general behavior of the fish groups was roughly similar with either full or partial covers, indicating that both were seen by the fish as a refuge. Interestingly, the development of the shelter-seeking behavior was progressive. It took several weeks for the fish groups in the sheltered tanks to show this clear shelter-seeking pattern, and different factors could have been involved in the development of this behavior. A plausible explanation for the time taken for the development of shelter seeking behavior relates to the need of a process of learning and/or adaptation to the presence of the shelter and to its potential use as a refuge. An alternative explanation is that fish only develop this behavior at a certain developmental stage and the shelter-seeking behavior is more related to fish age/size than to an adaptation process. It has been shown that boldness and shelter use can be affected in fish by stage/size dependent-motivation for feeding (Brown and Braithwaite, 2004). Finally, the social environment within the fish rearing units might also have affected the extent and speed of development of this behavior. In this regard, fish shelter-seeking behavior is known to be affected by the social environment (Näslund et al., 2013). Fish numbers inside the tanks were reduced at different times during the experimental period and for example, there were 110 fish per tank when the sheltering behavior was clearly observed, while there were 160 fish when the shelter-seeking behavior was first assessed. The reduction in fish numbers might have had an effect on the ability of the juveniles to be aware of their surroundings or to feel the need to seek for shelter. The importance on social environment and fish developmental stage on sheltering behavior has not been explored in salmonids under captivity and should be the focus of future studies, particularly given the strong shelter-seeking behavior observed in this study.

Different stress protocols were applied to both sheltered and non-sheltered fish during the experimental period. Acute stress protocols involved sampling fish before, and at two times after stress, while in the repeated stress trial fish were only sampled at a single point post-stress. The post-stress sampling point at 45 min aimed to capture the post-stress cortisol release peak, expected to occur at around that time in juvenile

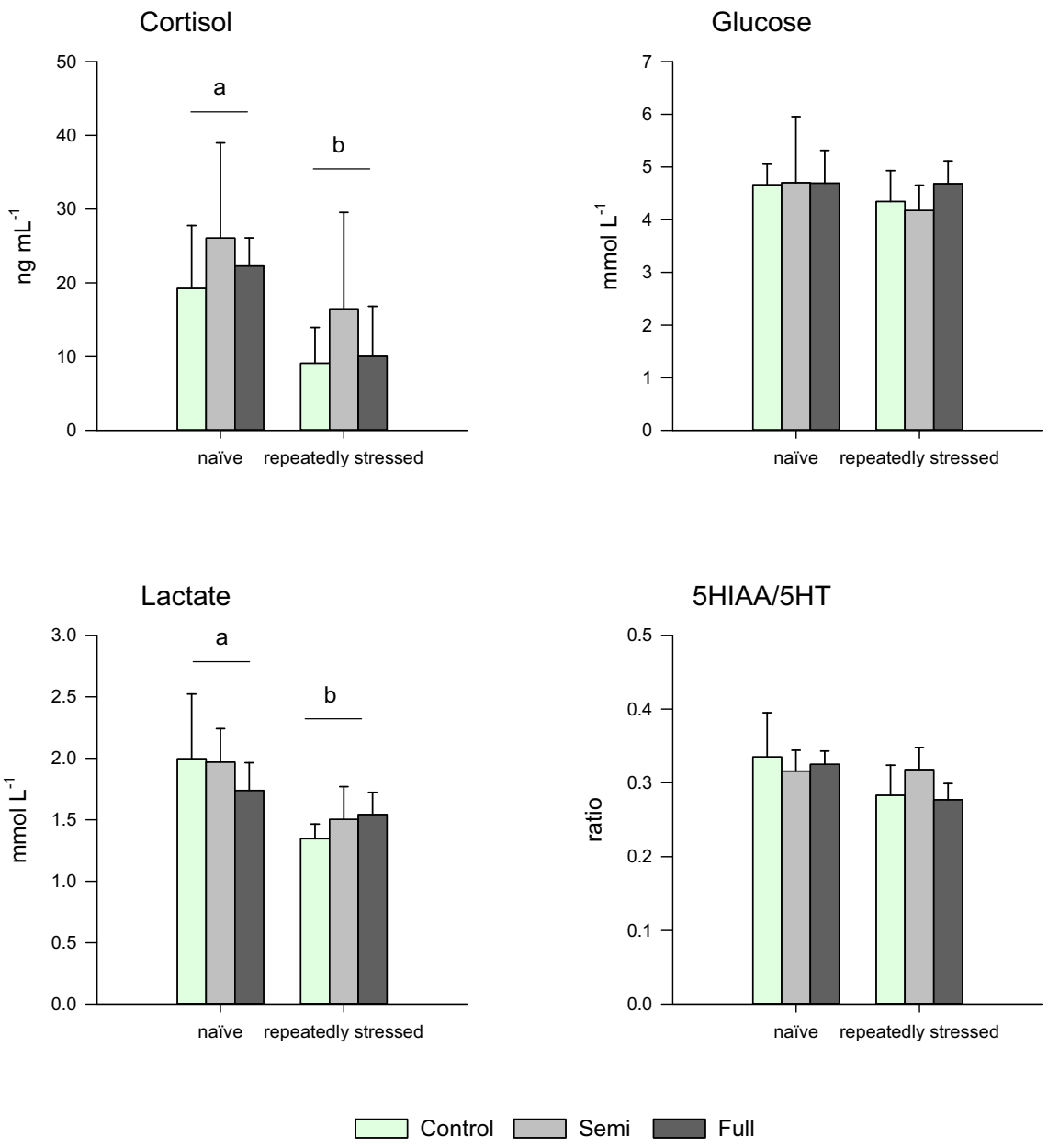

Fig. 3. Cortisol, glucose, lactate and telencephalic serotonergic ratio of the fish in trial 2. Fish were reared for 6 to 8 weeks in the presence of partial (Semi) or complete (Full) submerged screen shelters, while Control fish were reared in empty tanks. Fish were then exposed for one week to a daily stressor consisting in an acute reduction of water level in their tanks. Fish were sampled 45 min after stress start, on both the first and last stress episodes. Data represent the mean and SD of n = 3 tanks. Different letters indicate significant differences between Näive and Repeated Stress groups.

rainbow trout (Barton, 2002; Gesto and López-Patiño, 2013). A second sampling point at 120 min post stress was intended to provide information about the extent of recovery of the fish from the acute stress protocols. We hypothesized that the presence of a shelter might help the fish to have some perception of control over the stressors (Cerqueira et al., 2021), and that would result in stress responses of lower magnitude and/or faster recovery. However, no shelter-induced effects were found in any of the stress trials in terms of plasma cortisol and glucose, pointing to a similar level of experienced stress in both control and sheltered fish. In both acute stress trials, cortisol levels were elevated (at 45 min post stress) and then recovered (120 min post stress), and this pattern was not affected by the shelters. The transient cortisol response and its small magnitude (together with the lack of effects on plasma glucose), compared to other studies in rainbow trout of similar size (Auperin and Geslin, 2008; Barton and Peter, 1982), suggest that the stress experienced by the fish was mild. In the repeated stress trial, results showed a decrease in post-stress cortisol and lactate levels in fish repeatedly exposed to the stressor with respect to the fish exposed to the stressor only once, which might be reflecting an habituation/desensitization response (Cyr and Romero, 2009). The shelters did not alter post stress marker levels or the effects of the repeated exposure to the stressor.

In spite of the lack of differences in cortisol and glucose responses to stress, the plasma levels of lactate were altered by the presence of shelters. Plasma lactate is mostly originated as a product of the anaerobic metabolism in the muscle, which is increased under activities demanding a higher muscular effort. Those include, for example, the behavioral activation that often occurs during acute stress, and thus, lactate is frequently used as a metabolic indicator of stress (Milligan and Girard, 1993; Pankhurst, 2011). In the first acute stress challenge, an

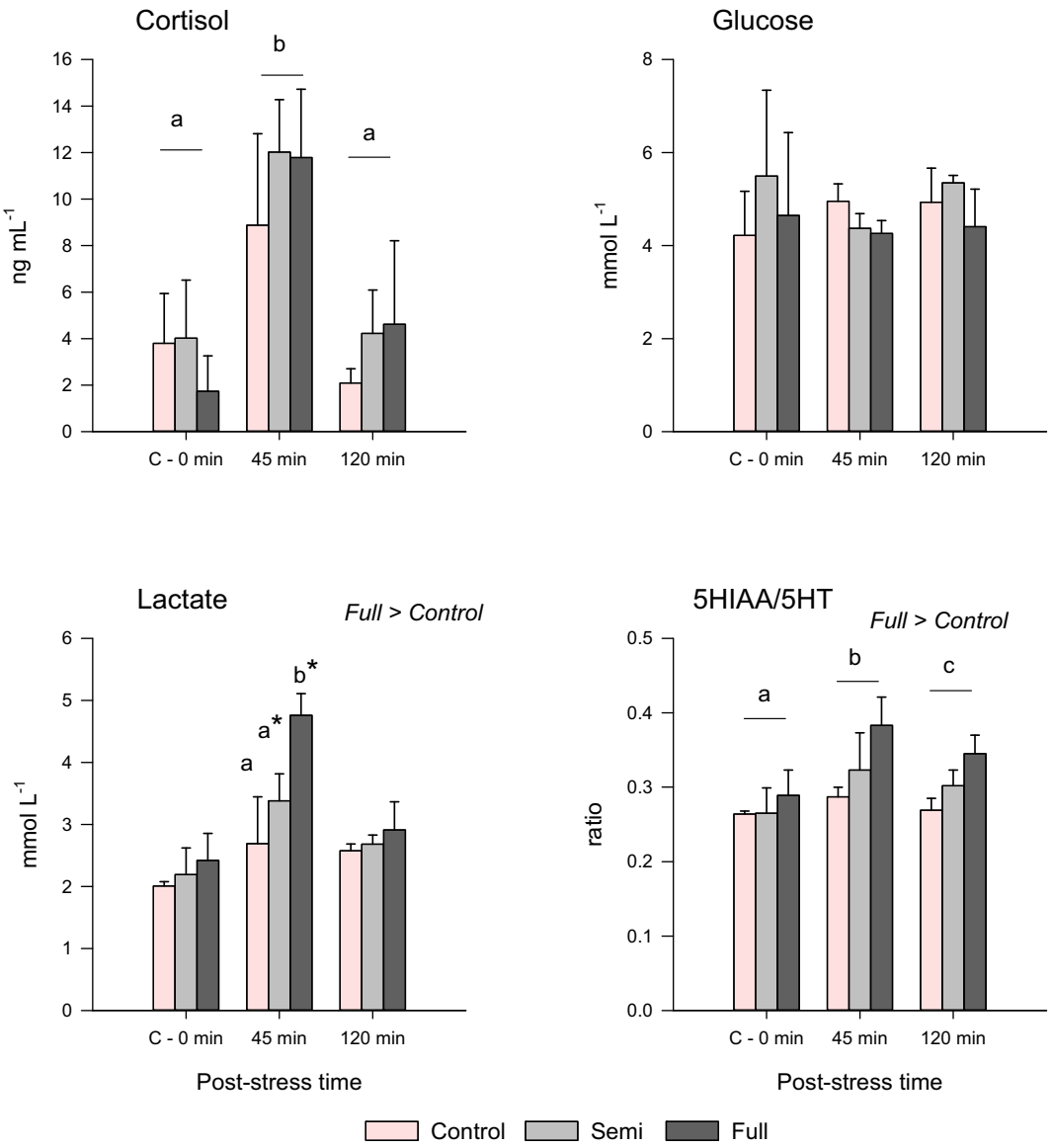

Fig. 4. Cortisol, glucose, lactate and telencephalic serotonergic ratio of the fish in trial 3. Fish were reared for 10 weeks in the presence of partial (Semi) or complete (Full) submerged screen shelters, while Control fish were reared in empty tanks. Fish were then exposed to an acute reduction of water level in their tanks followed by chasing stress for 1 min, and then sampled at 0 min (Control), 45 min or 120 min after stress. Data represent the mean and SD of n = 3 tanks. Different letters indicate significant differences among shelter groups within sampling times. Asterisks indicate differences versus controls (0 min) within shelter groups. Shelter-related main effects are indicated in insets.

overall reduction of lactate levels was observed throughout the experiment in the sheltered groups, maybe as a result of a reduced average activity of the fish. On the second acute stress trial later on, however, lactate levels were no longer reduced in sheltered groups; on the contrary, lactate response to stress was higher in the presence than in the absence of shelters, suggesting a higher behavioral activation of the fish upon exposure to the stress protocol. Even when this higher lactate did not occur along with a significantly larger cortisol response, it points to a higher startling/behavioral response of the sheltered fish during the stress protocol, maybe because the fish were forced to abandon the shelters due to the reduced water levels, and were then exposed to the chasing net. By being forced out of the shelters they lost any extent of controllability they might have had (Cerqueira et al., 2021), potentially contributing to a larger startling/behavioral response to the stressor than fish used to live without shelters. Interestingly, the brain serotonergic data seem to support this hypothesis. In the telencephalon of the fish under full covers, serotonergic activity, estimated using the turnover ratio between the metabolite 5-HIAA and serotonin (Winberg and Nilsson, 1993), was stimulated to a higher extent than in the non-sheltered fish upon exposure to the stressor. Serotonergic activity is consistently stimulated in certain brain areas upon exposure to stress in vertebrates (Dinan, 1996; Gesto et al., 2018), and is believed to participate in early stages of perception/recognition of stress by the central nervous system (Chaouloff et al., 1999; Gesto and López-Patiño, 2013; Puglisi-Allegra and Andolina, 2015). An increased startling response of sheltered groups was not observed in the first acute stress trial, neither in lactate levels, nor in telencephalic serotonergic activity. This could be related to the fact that fish were not showing a clear sheltering behavior at that stage, but likely also because the stressor applied in that first trial did not force the fish to abandon the protection of the shelters (since it consisted in waving the net just around and above the shelters). Altogether, these data suggest that, paradoxically, the presence of shelters might be counter-productive, in terms of stress controllability, if a disturbance forces the fish to leave the shelters, highlighting the need to

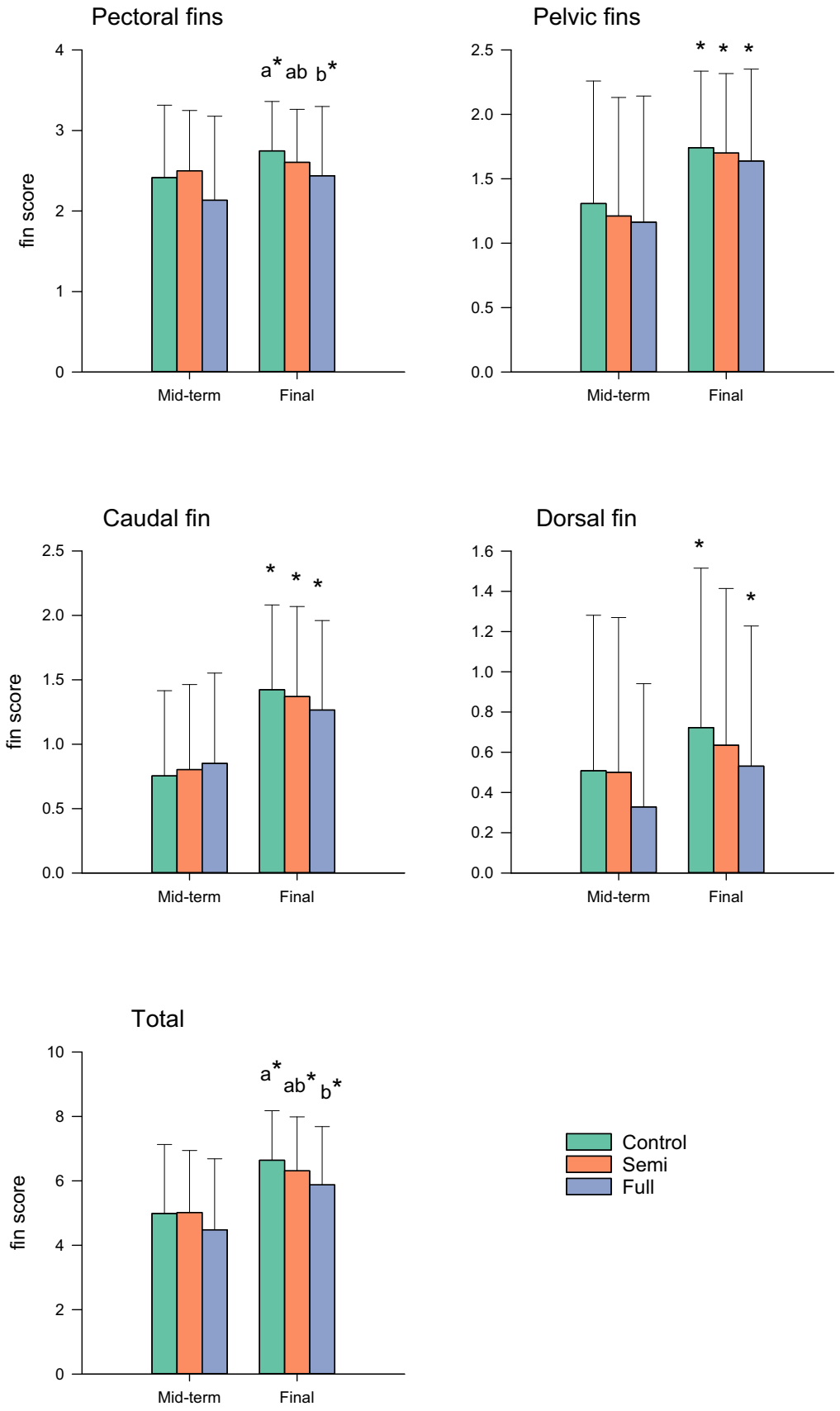

Fig. 5. Bar plots showing the pectoral, pelvic, caudal and dorsal fin damage scores, as well as the total fin damage scores of the different groups at mid-term and at the end of the experimental period. Different letters indicate significant differences within a specific sampling time (K—W). Asterisks indicate sampling time differences within shelter groups. Data represent the mean and SD of $n = 60$ (Mid-term) or $n = 156$ (Final) fish.

Table 3
P-values and significant effects (highlighted in bold) in fin damage scores.

	Effect of time (Mid-term vs Final)						Effect of Shelter (C vs Semi vs Full)			
	Control	*Effects*	Semi	*Effects*	Full	*Effects*	Mid-term	effects	Final	effects
Pectoral	**0.001**	*Final > Mid-term*	0.304	–	**0.027**	*Final > Mid-term*	0.105	–	**< 0.001**	*Control > Full*
Pelvic	**< 0.001**	*Final > Mid-term*	**< 0.001**	*Final > Mid-term*	**< 0.001**	*Final > Mid-term*	0.736	–	0.467	–
Caudal	**< 0.001**	*Final > Mid-term*	**< 0.001**	*Final > Mid-term*	**< 0.001**	*Final > Mid-term*	0.735	–	0.071	–
Dorsal	**0.040**	*Final > Mid-term*	0.167	–	**0.022**	*Final > Mid-term*	0.369	–	0.087	–
Total	**< 0.001**	*Final > Mid-term*	**< 0.001**	*Final > Mid-term*	**< 0.001**	*Final > Mid-term*	0.253	–	**< 0.001**	*Control > Full*

Effects of time were assessed by Mann-Whitney U tests
Effects of Shelter were assessed by Kruskal-Wallis tests

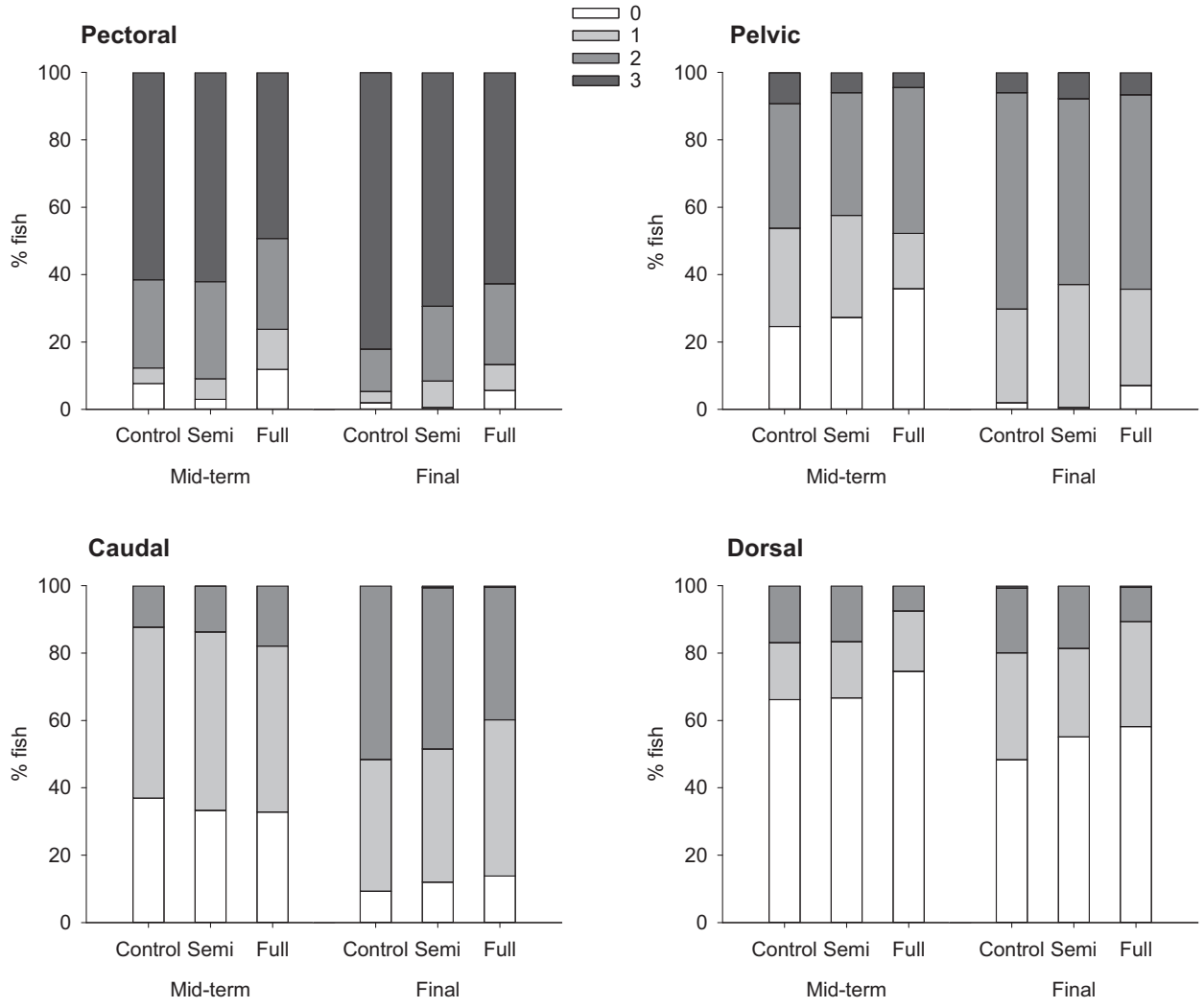

Fig. 6. Distribution of fish individuals of each shelter group among the different damage score categories for each fin(s), at both mid-term and at the end of the experimental period. Damage categories ranged from 0 (no damage) to 3 (severe damage). Data was calculated as % of individuals among the total fish tested for fin damage in each shelter group (n = 60 per group for *mid term* and n = 156 per group for *final*).

finely adjust the characteristics of the shelter to particular species and rearing conditions (Jones et al., 2021; Saraiva et al., 2021).

Fish fins are sensitive appendages (Hardy and Hale, 2020) and fin damage is an important threat for fish welfare (Hoyle et al., 2007; Weirup et al., 2021). The etiology of fin damage in captive fish is not fully understood, but potential causes include agonistic behavior (nipping), abrasion against rearing unit's surfaces, infections or stress (Ellis et al., 2008). Shelters can provide refuge for fish individuals to avoid confrontation with conspecifics but, at the same time, they can generate competition to occupy and/or defend the sheltered area. Therefore, it was expected that the type of effects of the shelters on fin damage might depend on the social environment in terms of density/fish numbers, which is known to affect agonistic behavior in trout (Adams et al., 2007; Gesto, 2019; Laursen et al., 2015). In the conditions tested in this study, the extent of damage to the fins increased in all shelter groups during the experimental period, in all evaluated fins (pectoral, pelvic, dorsal, caudal), with the exception of pectoral and dorsal fins in the Semi group, in which the increase was not statistically significant. The full shelters showed some protective effect in terms of fin damage, which was observed as a reduced damage score in this group in the

pectoral fin and in the total fin damage score, and as a reduced percentage of fish showing severe damage in the pectoral fin in the Full group. However, the size of the effect was relatively small, an 11–12% reduction in the average scores, and the only significant reduction was found in the pectoral fin, while the dorsal and caudal fins are often the most damaged during agonistic behavior in salmonids (Turnbull et al., 1998). In this study, the fin damage scores of the dorsal and caudal fins in the Full group showed a trend towards lower values than in the Control: Fin damage scores were 26% and 11% lower in the Full than in the Control group, for the dorsal and caudal fins (and *p*-values for shelter effects on those fins were $p = 0.087$ and $p = 0.071$, respectively). Altogether, the fin data show that the full shelters likely reduced aggression among the fish to some extent. An alternative (or complementary) view, is that full shelters could reduce the general swimming activity of the fish, thus reducing fin damage potentially caused by stochastic contact with tank walls (and shelter walls, which contained less edges than the partial shelters) and other fish. A few previous studies in fish reported positive effects of shelters on fin damage (Arechavala-Lopez et al., 2019; Näslund et al., 2013; Näslund and Johnsson, 2016), sometimes affected by the feeding levels rations level (Persson and Alanärä, 2014), but also lack of effects (Johannesen et al., 2018). Thus, the effects of shelters on fish fin condition likely depend on species, type of shelters, and on other factors potentially affecting agonistic behavior such as fish size or developmental stage, feeding rations and the social environment (fish numbers and stocking density).

In summary, collected data showed that the simple PVC screen shelters tested in this study had neither positive nor negative effects on the juvenile's growth performance or their ability to cope with acute and repeated stressors. However, there was some improvement of fin condition in fish from the Full group. Furthermore, the fish in the sheltered groups developed during the experimental period a very marked shelter-seeking behavior when exposed to external disturbance. This highlights the need to further investigate the potential welfare benefits of shelter availability for rainbow trout, particularly when no deleterious effect in growth performance, external damage or any other aspect were found in this study, which demonstrate no important disadvantages related to shelter presence. Preference, and providing animals what they prefer/like, is an important component in animal welfare (Fraser and Matthews, 1997; Kirkden and Pajor, 2006), and it makes an inherent part of the feelings-based definitions of welfare (Bovenkerk and Meijboom, 2013; Volpato, 2009). Different intrinsic or extrinsic factors may affect the value of shelter availability for fish welfare needs, including fish species, age/developmental stage, domestication level, stocking density, type of shelter, and timing and duration of shelter availability. Fish personality/individuality (Castanheira et al., 2017) could be also a very important factor determining the use of the shelters by different individuals, and determining the benefits that fish of different stress-coping style, i.e. proactive or reactive, can get from them. However, current knowledge about the influence of all those factors on the effects of shelters (or environmental enrichment, in general) is very limited (Arechavala-Lopez et al., 2021; Jones et al., 2021). Further studies are needed to precisely quantify the effects of shelter presence on fish physiology, behavior and welfare in relation to those factors, and to investigate how aquaculture-like conditions in terms of fish numbers and stocking densities might influence those effects.

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Credit Statement

Statement about the contribution of each author.

Manuel Gesto: Conceptualization, Formal analysis, Investigation, Writing - Original Draft, Visualization.

Alfred Jokumsen: Conceptualization, Resources, Writing - Review & Editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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