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Aquaculture Research

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Journal:	*Aquaculture Research*
Manuscript ID	ARE-OA-19-Dec-1194
Manuscript Type:	Original Article
Date Submitted by the Author:	06-Dec-2019
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Keywords:	fish, organic, behavior, welfare, aquaculture, trout

Moderate stocking density does not influence the behavioral and physiological responses of rainbow trout (*Oncorhynchus mykiss*) in organic aquaculture

Running title: Stocking density and organic aquaculture

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Abstract

Welfare in farmed fish got particular attention during the last decades from both governmental and public sides. In aquaculture context, welfare concerns are mainly related to handling procedures, water quality and stoking densities. In Europe, authorities had to clarify the threshold limits of stocking densities to maintain fish good welfare, including for organics aquaculture through the EC regulation 710/2009. However, effects of stocking density on fish welfare are complex and sometimes contradictory. Moreover, there is a lack of knowledge about the impact of density on fish welfare in organic aquaculture. Thus, the aim of the study is to asses welfare state of rainbow trout (*Oncorhynchus mykiss*) at two initial stocking densities (low density, LD: 12 kg/m^3 and high density, LD: 17 kg/m^3) fed using organic feed by combining the monitoring of growth performances, behavior (swimming activity) and physiological indicators (i.e. cortisol, glucose, lactate, hematocrit, red blood cellule count and lysozyme). At the end of experiment, the stocking density reached 21 kg/m^3 and 30 kg/m^3 for the LD and HD respectively. Overall, growth performances, swimming activity and level of physiological indicators of stress and welfare were similar between HD and LD over the experiment duration. To conclude, we observed no alteration of fish welfare between the two stocking densities monitored. This study suggests that a final stocking density of 30 kg/m^3 can be considered for organic aquaculture of rainbow trout respecting welfare.

KEYWORDS

fish; organic; behavior; welfare; aquaculture; trout.

1 | INTRODUCTION

The welfare of farmed fish got particular attention from governmental, public and scientific sides. On the one hand, consumer concerns about fish welfare has grown during the two last decades (Ellis et al., 2002; Solgaard & Yang, 2011). On the other hand, scientists also pointed out the possible current issues in fish welfare (Conte, 2004; Huntingford et al., 2006; Ashley, 2007). As a consequence, in Europe, authorities adopted legislation to ensure fish welfare over all life duration during farming (European Food Safety Authority, 2009).

However, welfare in fish is quite difficult to define and gathers debates because talking about welfare means that fish possess all needed cognitive and emotional capacities linked to the mental state of individual (Huntingford & Kadry, 2008; European Food Safety Authority, 2009). The definition of welfare can be based on three main points which are linked to feeling, physiological functions and "nature" as explained by (Huntingford et al., 2006). Taking into consideration these three points, we can consider that an individual displays a good welfare state when is freedom from hunger and thirst, discomfort, pain, injury, disease, fear and distress, biological functions run well and fish is free to express normal behavior in captivity as it will be in the wild (The "five freedoms"; (Brambell Comittee, 1965)).

Moreover, the welfare state of fish is closely linked to the stress response (Prunet et al., 2012). In fish, exposure to a stressor trigger the activation of the hypothalamo-pituitary-interrenal axis (HPI), which is involved in the production and release of and cortisol in cardiovascular system (Mommsen, Vijayan & Moon, 1999), mediating the physiological and behavioral responses to cope with stress factors (Sadoul & Vijayan, 2016; Schreck & Tort, 2016). For instance, cortisol release modulates energy mobilization and changes in hematological features (*e.g.,* hemoglobin, hematocrit, leukocrit) and immune functions features (lysozyme activity) (Barton, 2002). Therefore, on one hand, measuring cortisol and other physiological parameters linked to metabolism, hematology and immunity are good stress and welfare indicators (Barton, 2002; Sadoul & Geffroy,

2019). In the other hand, behavior is also a good indicator to quantify fish welfare, as reviewed by (Martins et al., 2012), Indeed, the swimming and feeding activities, position in the water column, aggressive and group behaviors are important to quantify the fish welfare in aquaculture context (Martins et al., 2012). Mostly, the swimming activity is considered in the perspective of the energy balance evaluation (Cooke, Chandroo, Beddow, Moccia & McKinley, 2000; Mckenzie, 2011; Nelson, 2016), and thus important when regarding the wellbeing of fish in their environment.

The mains issues concerning welfare in fish include water quality, stoking density or handling procedures linked to transport and slaughtering (Conte, 2004; Ashley, 2007; Lembo, Carbonara, Fabris, Manfrin & Zupa, 2019). Many studies have reported harmful but also some positive effects of high stocking density on the physiological and behavioral indicators of welfare in several fish species (Ellis et al., 2002). For instance, in sea bream (*Sparus aurata*), high stocking density induces a chronic stress for fish which is translated by an increase of cortisol, hematocrit and hemoglobin concentrations and red blood cell count in blood (Montero, Izquierdo, Tort, Robaina & Vergara, 1999). In European sea bass (*Dicentrarchus labrax*), the muscular activity, monitored using electromyogram, was shown to be higher in fish reared in high stocking density suggesting a higher energetical cost of life (Carbonara *et al.*, 2015). At the opposite, (van de Nieuwegiessen, Boerlage, Verreth & Schrama, 2008; van de Nieuwegiessen, Olwo, Khong, Verreth & Schrama, 2009) reported that low densities may increase aggressive behavior leading to increase in skin lesions in African catfish (*Clarias gariepinus*).

In the case of rainbow trout (*Oncorhynchus mykiss*), the effects of stocking density are also not always clear and sometimes contradictory as reviewed by (Ellis et al., 2002). The majority of studies reviewed by these authors pointed out negative effects of density on welfare, including about production, nutritional status and health but rarely highlighted changes of stress indicators (e.g., cortisol, glucose, lactate). In the same way, equal number of studies highlighted increase mortality or no change related to stocking density. At the opposite, some authors shown an increase

of mortality at lower stocking density (e.g (Papoutsoglou, Papaparaskeva-Papoutsoglou & Alexis, 1987; Bagley, Bentley, & Gall, 1994)). It important to note that, in rainbow trout, low stocking density may increase the number of severe social interactions and thus disease susceptibility which can impact growth and survival (Pottinger & Pickering, 1992). Thus, the choice of the appropriate stocking density is essential to ensure the best health and welfare conditions for fish while best production performances.

During the last years, fish production from organic aquaculture has rapidly increase (Gambelli, Naspetti, Zander, & Zanoli, 2019). Standard to reach organic certification are obviously linked to diet composition, but also to waste production related to the production systems and welfare state of fish (Gould, Compagnoni, & Lembo, 2019). According to the EC regulation 710/2009 relative to organic production of fish, the stocking density is an important standard that producers have to take into account during the rearing. In rainbow trout, to be certificated as "organic aquaculture", the final stocking density must not exceed 25 kg/m^3 in freshwater and 10 kg/m^3 in marine water. There is, however, a lack of data regarding the behavioral and physiological state of fish related to welfare in response to stocking density using organic standard, including in rainbow trout.

Thus, the aim of the study is to combine both physiological and behavioral indicators, as well as growth performances, to monitor welfare of rainbow trout using organic standard reared at two different stocking densities. To assess the welfare state of individuals, a holistic perspective was used. Indeed, on one hand, the whole organism response was monitored using swimming activity and growth performance. On the other hand, blood samples were taken in order to measure the physiological stress (i.e. cortisol, glucose, lactate, hematocrit, red blood cellule count and lysozyme)

2 | MATERIALS AND METHODS

2.1 | Ethics Statement

The experiment was performed in accordance with Italian national legislation (D. lgs. 26/2014) and EU recommendation (Directive 2010/63/EU). The protocol was approved by the Committee on the Ethics of Animal Experiments of COISPA (Italian Ministry of Health 15/2015-UT). All fish manipulations (morphometric measurements, blood samples, surgical implantations) were performed on fish that were completely anaesthetized (stage 4: loss of reflex activity and no reaction to strong external stimuli, as reported by (Iversen, Finstad, McKinley, & Eliassen, 2003) with a 50 mg L^{-1} clove oil solution), minimizing pain and discomfort. The survival rate after the manipulations (morphometric measurements, blood sample and surgical implantations) was 100%, and all efforts were made to minimize suffering.

2.2 | Fish holding conditions, fed regime and experimental procedures

Rainbow trout (*Oncorhynchus mykiss*) used in the experiment (n=488) were provided by commercial hatchery ("Troticoltura Bassignana"; Cuneo, Italy) and transferred at the experimental facility of the University of Torino (Italy).

After acclimatization, the fish were randomly distributed into 6 tanks of 3 m^3 at two stocking densities (low density, LD: 12 kg/m^3 and high high density, HD: 17 kg/m^3) with three replicates per stocking density. The weight of fish was 401.1 ± 5.3 g (n=197) and 374 ± 5.2 g (n=291; mean ± s.e.m) for LD and HD respectively. Fish were reared in fresh water at a constant temperature of 13 ± 1°C. A continuous photoperiod of 12L:12D was imposed for the whole duration of the experiment. In the two experimental groups, the same water exchange condition was performed (i.e. six volumes per day) in order to maintain an oxygen concentration > 80 % during the all experiment duration.

During the experiment duration, rainbow trout were fed with a ratio equal to 0.8 % of the biomass in the tank by using organic feed (Emerald Trout 60, Skretting, Italy). Feed used in the study complies with standards for feeds set by the Soil Association for organic farming and trout production. Proximate composition of feed is shown in **Table 1.**

The experiment lasted 97 days, including two blood samples at the start and the end, to measure stress and welfare physiological indicators, six biometries to measure morphological parameters (from B1 to B6) and the monitoring of swimming activity using accelerometers tags (**Figure 1**). All the procedures are detailed below in the specific sections.

2.3 | Growth performances

Growth performances were measured six times during the whole duration of experiment at t=16, 41, 62, 73,90 and 97 days corresponding to B1, B2, B3, B4, B5 and B6 respectively (**Figure 1**). During each biometry of the experimental period, the biomass of each tank (n=3 per density; kg) was assessed and the mean of fish weight inside each tank was estimated by dividing the total biomass of tank by the number of fish (g). The Specific growth rate (SGR) was calculated for each tank, based upon the mean fish mass as follow:

$$SGR = 100 * \frac{(\ln(W_{B6}) - \ln(W_{B1}))}{T}$$

where W is the mean weight (g) of fish per tank respectively at the biometry B6 (W_{B6}) and the biometry B1 (W_{B1}) and T is the number of feeding days between the two biometries. The feed conversion ratio (FCR) was calculated as the ratio of the feed supplied (kg of dry weight) per biomass of weight gained (kg). The protein efficiency ratio (PER) was calculated as a ratio of the total biomass for each tank per the total proteins assumed.

2.4 | Swimming activity

166 At t=16 day and, a total 18 fish were surgically implanted with tailbeat accelerometer tags (3 fish
167 per tank per stocking density randomly selected) to measure swimming activity (i.e., acceleration,
168 m/s^2). Fish randomly selected had similar standard length (SL, cm) and weight (g) between stocking
169 densities (mean ± s.e.m; SL: 291.22 ± 6.25 and 278.11 ± 3.4 cm; weight: 416.33 ± 27.02 and
170 380.11 ± 15.88 g for HD and LD respectively). The tags used were V9Aacoustic accelerometer
171 manufactured by VEMCO programmed to measure, with a sampling rate of 10 Hertz (10
172 measurements/s). The tag ID and the coded values corresponding to the acceleration were stored in
173 the memory of submergible acoustic receivers (Vemco VR2W). The animal's acceleration signal is
174 measured in terms of $m\ s^{-2}$ and is a vector quantity that is a result of measuring acceleration on 2
175 axes (X,Z) by a tail-beat algorithm. Fish were fasted 24 h before implantation and were
176 anaesthetized with a 50 ppm hydroalcoholic clove oil solution as described above. The same clove
177 oil solution was used to continuously irrigate gills during the surgery. The tag is introduced in the
178 abdominal cavity throughout a 1.5 cm incision in the body cavity (Lembo, Carbonara,
179 Scolamacchia, Spedicato, & McKinley, 2007) (**Figure 2**). Tags recorded the data of the acceleration
180 for 33 days (from the days 29 to 61 of the experiment, see **Figure 1**).

181

182 **2.5 | Physiological and morphometric measures**

183 For the procedures described below (blood sample and morphometrics measurement), fish were
184 gently caught from their rearing tank and anesthetized using a clove oil solution at 50 ppm. Within
185 5 min after anesthesia, the blood was taken from the caudal vein using a heparinized syringe at the
186 beginning of the trial (n= 5 fish per tank; i.e. n=15 per stocking density) and at the end (n= 10 fish
187 per tank; i.e. n=30 per stocking density). the same time points, morphometric measures (total
188 length, TL: at the beginning; TL, total, viscera, liver and gutted weight: at the end) were in n=10
189 fish per tank (n=30 per stocking density) to estimate the hepato-somatic index (HSI) and the

190 visceral-somatic index (VSI). The HSI was calculated as the ratio of fish liver weight (g) per gutted
191 fish weight (g). The VSI was calculated as the ratio of viscera weigh (g) per gutted fish weight (g).
192 Blood sampling were performed to measure physiological stress and welfare indicators (cortisol,
193 glucose, lactate, hematocrit, red blood cellule count and lysozyme; **Figure 1**) as described in
194 (Carbonara et al., 2019b) and below.
195 Briely, hematocrit (Hct) was determined using a heparinized micro-hematocrit tube, filled directly
196 from the syringe needle and centrifuged (15000 g for 3 min), and immediately read. Haematocrit
197 values were expressed as the percentage of the red blood cells on the whole blood volume. The red
198 blood cells (RBCC) were counted in a Burker counting chamber under a light microscope (Nikon
199 400E, Japan). Plasma cortisol concentration was determined using a commercial ELISA (enzyme-
200 linked immunosorbent assay) kit for microplate readers (k = 450 nm; InterMedical, Italy). Plasma
201 glucose and lactate concentrations were determined using commercial kits 17630H and 17285 for
202 glucose and lactate respectively (Sentinel, Italy) based on the enzymatic colorimetric Trinder
203 reactions (GOD/PAP for glucose and PAP for lactate). Plasma lysozyme concentration was
204 measured using turbidimetric assay modified for a microplate reader (Carbonara et al., 2010,
205 2019a).
206
207 **2.6 | Statistical analysis**
208 Statistical analysis where performed with the open source R software (R Core Team, 2018) and
209 were carried out at the 95% level of significance. Values are expressed as mean ± s.e excepted
210 otherwise mentioned. Normality check of the data was carried out using Shapiro test and
211 appropriate statistical test was then performed. Thus, to analyze the SGR, FCR, PEF, VSI, we
212 performed Wilcoxson rank sum, while we performed t-test to compare the HSI values between the
213 two stocking densities.
214 During the monitoring of swimming activity (from t = 29 to t = 61), the acceleration was first
215 averaged for each days of monitoring (n=33) depending on stocking density and linear regression

was applied to evaluate the dynamic of acceleration over experimental duration depending on stocking density. To compare swimming activity between the two stocking densities, a generalized linear mixed model (GLMM) was applied using the stocking densities (HD and LD) and the period of the day (light and dark) as fixed factors and fish ID as random factor using the package *lme4* (Bates, Mächler, Bolker, & Walker, 2014). Since acceleration is strictly positive and continuous, we used a GLMM with Gamma distribution family and logarithmic link. The GLMM was followed by Tukey HSD post-hoc test and marginal means of the models were estimate using the package *emmeans* (Lenth, Singmann, Love, Buerkner & Hervé, 2019). Pseudo R^2 of the model was calculated using the package the *MuMIn* (Barton, 2019). Visual inspection of the residuals revealed no violation of the statistical assumptions by the model. It is important to note that one individual from LD was removed from the statistical analyses because of accelerometer tag acquisition defaults (resulting to a number of n=17 fish; n=9 for HD and n=8 for LD).

Finally, to analyze the physiological parameters (i.e. cortisol, glucose, lactate, hematocrit, red blood cellule count and lysozyme), a two-way ANOVA was carried out including the stocking densities (HD and LD) and the sampling time (i.e. beginning and end of the experiment) as fixed factors. The ANOVA was followed by Tukey HSD post-hoc test.

3 | RESULTS

3.1 | Growth performances

At the end of the experiment, the stocking densities reached 21 and 30 kg/m^3 for LD and HD respectively. Body weight of rainbow trout over the duration of experiment from B1 to B6 is shown in **Figure 3**. At B1, the weight of rainbow trout was 374.4 ± 5.2 and 401.1 ± 5.3 g for HD and LD respectively. At the end of the experiment (B6), the weight of rainbow trout was 672.6 ± 8.4 and 682.6 ± 42.9 g for HD and LD respectively. The specific growth rate (SGR), the protein efficiency rate (PER) and the feed conversion rate (FCR) did not differ between HD and LD (Wilcoxon rank

sum test, p >0.05 for all). There is no more difference in the HSI and VSI between the two stocking densities (Wilcoxon rank sum test, p > 0.05 for both; **Table 2**).

3.2 | Swimming activity

Swimming activity (i.e. acceleration) was negatively correlated with the day of experiment ($p < 0.001$, $df = 31$; $R^2 = 0.41$) in the HD while there was no correlation between swimming activity and the day of experiment in the LD ($p > 0.05$; **Figure 4**).

Outputs of generalized linear mixed model (GLMM) to estimate acceleration as function of stocking densities and period of the day (i.e. days or night) are shown in **Table 3**. Stocking density has no effect on acceleration whereas both period of the day and the interaction between period of the day and density have effect on acceleration (**Table 3**). Rainbow trout has shown a clear diurnal pattern during the experiment (**Figure 5.a**). Indeed, both fish from HD and LD displayed higher activity during the light period than during the dark period (Tukey HSD, $p < 0.001$ for both stocking densities) while there is no difference in swimming activity between HD and LD whatever the period of the day (Tukey HSD, $p > 0.05$ for both stocking densities; **Figure 5.b**). For the day period, the marginal mean estimated by the model are 1.07 ± 0.02 and 1.05 ± 0.02 m/s², while during the night period are 0.65 ± 0.01 and 0.69 ± 0.01 m/s² for LD and HD respectively (**Figure 5.b**).

3.3 | Physiological parameters

Between the start and the end of experiment, all the physiological measured (i.e. cortisol, glucose, lactate, HCT, RBCC and lysozyme), has shown an increase concentration in plasma regardless of density (ANOVA, $p<0.001$ for all; **Figure 6**). However, density effect was observed only for HCT and RBCC (ANOVA, $p = 0.03$ and $p = 0.04$ for HCT and RBCC respectively). No interaction

between sampling time (i.e. start and end) and stocking density on the physiological parameters was observed expected a trend for lactate concentration (ANOVA, $p = 0.05$). Inside stocking density groups, post-hoc tests indicated that the concentration of all physiological parameters is higher at the end of experiment than at the beginning (HSD Tukey, $p < 0.05$) expected for cortisol concentration in LD (HSD Tukey, $p > 0.05$). However, there is no difference in the concentration of all physiological parameters measured between HD and LD at both start and end of the experiment (HSD Tukey, $p > 0.05$).

4 | DISCUSSION

Here, we evaluated the physiological and behavioral indicators of stress and welfare, as well as growth performances, of rainbow trout at two starting different stocking densities (LD: 12 and HD: 17 kg/m^3) feed using organic food. Stocking density is one of the most important factors able to affect fish welfare in aquaculture (Conte, 2004; Ashley, 2007; Lembo et al., 2019). Due to the recent increase of fish production from organic aquaculture (Gambelli et al., 2019), and the importance of fish welfare in this context, it is significant to address this question in the most important farmed fish species. A holistic approach, including the monitoring of biological performances, physiological and behavioral measurements, was adopted to address this question in rainbow trout.

At the end of the experiment, the stocking densities reached 21 and 30 kg/m^3 for LD and HD respectively. The growth performances recorded during the experimental period are lower than those reported in the literature for circa similar temperatures (e.g. 14°C, (McKenzie et al., 2012); 13°C, (Belforti et al., 2015). For instance, the SGR measured in the present study was 0.65 ± 0.12 for LD and 0.69 ± 0.04 for HD, while the SGR measured in (McKenzie et al., 2012) was 1.41 ± 0.04 at similar density. However, the fish size, as well the feed regime were different between this study and our, which can both clearly explain the divergence observed in term of growth

(McKenzie et al., 2012). Anyway, in the present study, we did not observe any difference between the two stocking densities for all the variables related to growth performances (i.e. SGR, FCR, PER, HSI, VSI), fed with organic regime. It is, however, important to note that the feed conversion rate is relatively higher in the HD compare to the LD (i.e. 1.16 ± 0.01 vs. 0.75 ± 0.01) without being significantly different each other. This suggests that the feed efficiency could be better in LD than in HD, even if the SGR is relatively similar between the two stocking densities (i.e. 0.69 ± 0.04 vs. 0.65 ± 0.12 for HD and LD respectively). The future studies, having a higher sampling size, may bring further insight about the trend about the FCR, which has been observed in the present study. Anyway, this lack of difference first shows that the HD investigated does not impair the growth of fish, and therefore suggests that the conditions of welfare are respected for the rainbow trout. Indeed, the growth performances are an integrative measure of fish welfare in aquaculture, but supplementary physiological and behavioral measures are, nevertheless, important to confirm the fish wellbeing and coping in their environment (Carbonara et al., 2019b).

As reviewed by (Martins et al., 2012), the swimming activity is an important behavioral indicator of fish welfare. In the present study, we observed a decrease of the acceleration of fish over the experiment in HD whereas the acceleration was relatively similar in LD. Interestingly, we observed similar pattern in sea bream where a decrease of acceleration was found only for fish reared at a stocking density of 30 kg/m^3 but not for fish reared at 15 kg/m^3 (Carbonara et al., 2019b). This could be explained by the fact that the progressive increase of fish density in HD limits the propension for fish to display higher acceleration. In both stocking densities, the rainbow trout shown diurnal activity pattern as previously well described in the literature ((Sánchez-Vázquez & Tabata, 1998; Bégout Anras & Lagardère, 2004); but see also (Cooke et al., 2000)). High stocking density may disrupt diurnal activity pattern of rainbow trout as reported by (Bégout Anras & Lagardère, 2004) starting at 80 kg/m^3 but this is not the case for the stocking densities used in our experiment. Moreover, we did not observe any significant difference in swimming activity level

between the two stocking densities either during the day or night. The swimming activity, including the acceleration, is linked to metabolic functions and muscular activity in fishes (Cooke et al., 2000, 2004; Mckenzie, 2011; Zupa, Carbonara, Spedicato & Lembo, 2015), as well as in other species. The mean acceleration measured during the experimental monitoring corresponds to an oxygen consumption of 368.92 ± 57.11 and 361.80 ± 54.52 mg O^2/kg/h for fish from HD (n=9) and LD (n=8) respectively, which both are under the maximum metabolic rate of rainbow trout (703.32mg O^2/kg/h) for fish with similar size (W. Zupa, personal communication). These results suggest that in terms of oxygen demand and muscle activity, the densities reached are such that they do not generate critical conditions for the welfare and survival of the species in the farm. Indeed, a wide margin of energy is guaranteed for the rest of the non-basal vital functions, including reproduction, growth and various types of stress defense (Chabot, McKenzie & Craig, 2016; Norin & Clark, 2016).

From a physiological point of view, all the parameters investigated in the present study were in the normal range for this species and are reliable indicators of fish health, welfare and the ability to cope with stressors found in aquaculture context, including high stocking density (e.g., (Fevolden, Røed & Fjalestad, 2002; Huntingford & Kadry, 2008; Skov, Larsen, Frisk & Jokumsen, 2011)). In our study, the concentration of all physiological parameters monitored increased from the beginning to the end of the experiment suggesting higher level of stress for fish at the end of experiment. We, however, did not observe any difference between the two stocking densities suggesting that the higher stocking density (i.e. 17 kg/m^3 at the beginning) did not trigger a particular alteration of welfare state in rainbow trout compare to the lower stocking density (i.e. 12 kg/m^3 at the beginning). Cortisol is the major stress hormone in teleost fish, modulating a wide range of physiological functions, behavioral responses and ultimately reproduction and growth performances (Schreck & Tort, 2016). Overall, cortisol concentration does not particularly seem be affected by high density in rainbow trout (Kebus, Collins & Brownfield, 1992; Procarione, Barry & Malison,

1999; Ellis et al., 2002) but could be higher in very low density (10 kg/m^3) than in relatively high stocking density (80 kg/m^3) (North et al., 2006). The hematological parameters (*i.e.,* hematocrit (HCT), hemoglobin (Hb) and red blood cellule count (RBCC)) are relevant indicator of fish health status (Houston, 2004) and sensible to a wide range of stressors (LeaMaster, Brock, Fujioka & Nakamura, 1990; Carbonara *et al.*, 2015). Hematocrit is generally not altered for rainbow trout in high density (Ellis et al., 2002; Skov et al., 2011)) as we also reported in the present study. However, a lower RBCC level was reported in high density (100 kg/m^3) compared to low density (25 kg/m^3) for rainbow trout reared at high temperature of 19 °C (Skov et al., 2011). In the present study, we did not observe any difference in RBCC level between the two stocking densities, probably because our two stocking densities were not so different for triggering welfare disruption regarding to the RBCC level. Glucose and lactate are essential for the storage of energy reserves and therefore their availability upon stress (Barton, 2002; Polakof, Panserat, Soengas & Moon, 2012). The concentration of glucose has been also found higher in the blood of juvenile rainbow trout reared at low density compared to medium and high density (Procarione et al., 1999). In the case of density effects on welfare of rainbow trout, the variation of the lactate concentration was less studied even if crowding is known to induce an increase of the level of lactate in the blood (Yarahmadi, Miandare, Fayaz & Caipang, 2016). In our study, there is no difference on the lactate level between the two stocking densities, probably because the higher density was not high enough to trigger particular crowding. Finally, lysozyme is related to the innate immunity in fish (Saurabh & Sahoo, 2008) and has been found similar between the two stocking densities in our study. Lysozyme activity can vary in rainbow trout depending on the stocking density at certain points of the season as reported by (North et al., 2006), even if the activity is relatively similar between low and high densities. Taking together, these results suggest that the high stocking density in our experiment do not trigger a welfare issue for rainbow trout fed by organic food, as long as a good water quality is ensured as suggested by (North et al., 2006). It is, however, important to note, first, that other physiological parameters such as dopamine, noradrenaline and serotonin levels are also

366 important markers of the stress response and coping in fishes (Gesto, Lopez-Patino, Hernandez,
367 Soengas & Miguez, 2013; Gesto, López-Patiño, Hernández, Soengas & Míguez, 2015; Schreck &
368 Tort, 2016) and thus could show possible variation in response to stocking density. Second, even if
369 the basal concentration of cortisol in plasma was similar between the two stocking densities, it is
370 also possible that the response can vary upon stress exposure, either in the amplitude or recovery to
371 basal level (Tudorache, Schaaf & Slabbekoorn, 2013; Schreck & Tort, 2016).

372 In conclusion, we observed no alteration of fish welfare (both from behavioral and physiological
373 measurements) between the two stocking densities monitored. Moreover, the growth performances
374 were similar between the two stocking densities. Thus, this study suggests that a final stocking
375 density of 30 kg/m^3 can be considered for organic aquaculture of rainbow trout, respecting the
376 welfare according to the EC regulation 710/2009.

377

378 **ACKNOWLEDGMENTS**

379 This work was performed with the support of the Italian Ministry of Agriculture, Food, and Forestry
380 Policy (MiPAAF) (Action 2.2 for organic agriculture, BIOLFISH project). The authors would like
381 to thank Mr. Dario Sola, Dr. Paolo Montersino and Mr. Mario Colombano for fish care.

382

383 **DATA AVAILIBILITY STATEMENT**

384 Data will be made available on request.

385

386 **CONFLICT OF INTEREST**

387 The authors declared that they have no conflict of interest.

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537

TABLE 1. Proximate composition (% as it) and digestible energy (MJ/kg) of the feed used during the experiment (Emerald Trout 60, Skretting, Italy)†

Proximate content of feed	Percentage of content (%)
Crude Protein	38
Crude fat	26
Crude Ash	19
Phosphorous	1.6
Digestible energy	19.5

† Feed contains, fish meal and fish oil obtained from sustainable certified fishery byproducts; organic wheat, organic sunflower meal, vitamin and mineral mixture, antioxidants.

TABLE 2. Mean ± s.e of the performance parameters: specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), hepato-somatic index (HSI) and visceral-somatic index (VSI) between the two stocking densities (n=3 tanks per stocking density for SGR, FCR and PER; n=30 fish per stocking density for HSI and VSI).

Variables	High Density	Low Density
Specific growth rate	0.69 ± 0.04	0.65 ± 0.12
Feed conversion rate	1.16 ± 0.01	0.75 ± 0.01
Protein efficiency ratio	3.13 ± 0.23	3.22 ± 0.53
Hepato-somatic index	1.22 ± 0.06	1.17 ± 0.29
Visceral-somatic index	11.47 ± 1.42	10.76 ± 1.21

TABLE 3. Outputs of generalized linear mixed model (GLMM) for the acceleration as a function of stocking density (i.e. LD or HD) and period of the day (i.e. days or night). Reference factor level for the stocking density is LD and for the period is day. Pseudo R^2 of the model is 0.35. Significant factors are highlighted in bold.

Fixed effects				
	Estimate	Std. error	t value	p value
(intercept)	0.064	0.019	3.272	**0.001**
Density	-0.014	0.027	-0.531	0.596
Period	-0.493	0.002	-293.584	**<0.001**
Density:period	0.072	0.002	32.485	**<0.001**
Random effect				
	Estimate	Std. dev.	n	
Individual	0.003	0.55	17	
Residual	0.107	0.33		

FIGURE 1. The time-course of the experiment from the start to the end (i.e. corresponding to the two blood sampling points) including the biometrics points (B1, B2, B3, B4, B5 and B6). Grey part corresponds to the monitoring of swimming activity using accelerometers tags.

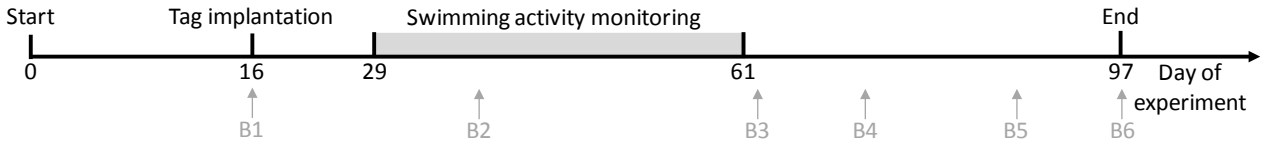

FIGURE 2. Surgical implantation of a V9A tailbeat accelerometer tag (Vemco) in the abdominal cavity of rainbow trout.

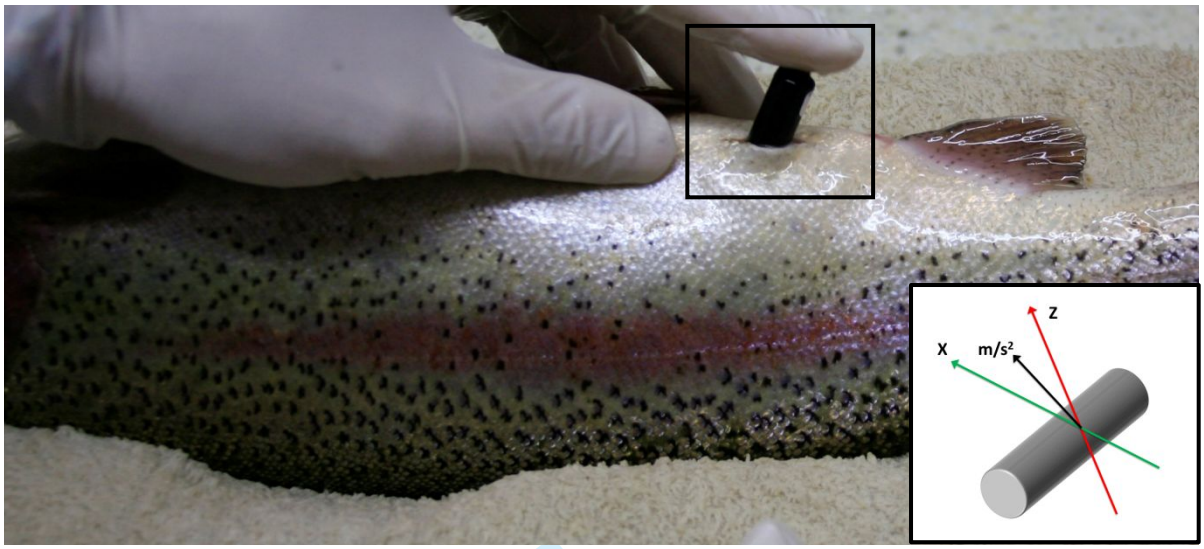

FIGURE 3. Mean ± s.e.m of body weight (g) for rainbow trout over experimental duration (from B1 to B6) for high density (HD, grey; n=3 tanks) and low density (LD, blue; n=3 tanks).

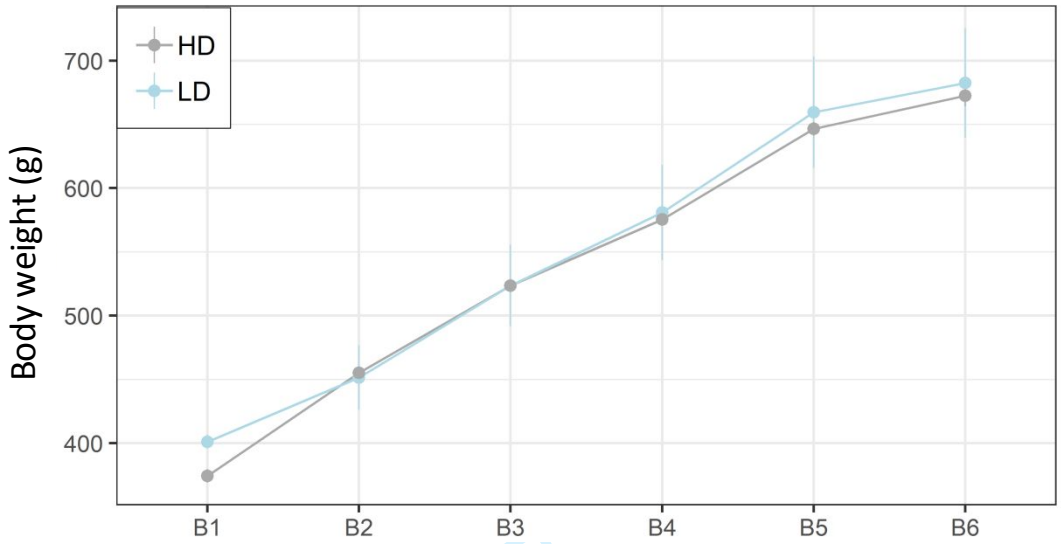

FIGURE 4. Relationship between acceleration (m/s^2) and day of experiment in **(a)** High density and **(b)** Low density during. The P value and associated R^2 are indicated for significant linear regression. Otherwise, NS (not significant) is indicated. The black line represents the significant linear correlation between acceleration and the day of experiment; the gray shape represents the 95% confidence interval.

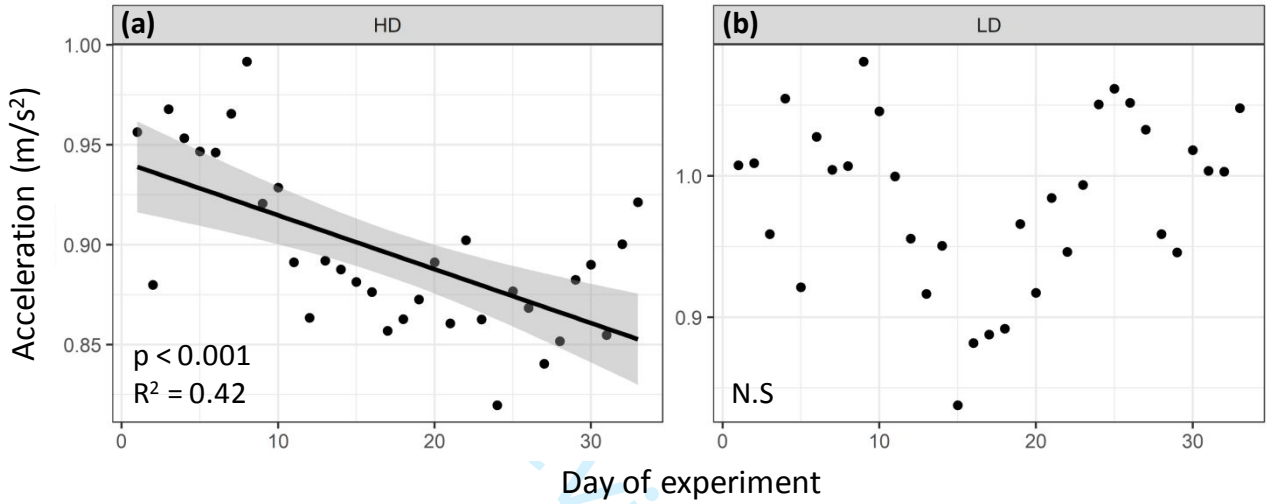

FIGURE 5. (a) Mean of acceleration (m/s^2) as a function of daily hour for high density (HD, grey; n=9) and low density and stocking densities (LD, blue; n=8); **(b)** Estimated marginal mean ± s.e. of acceleration (m/s^2) as a function of period of the day (i.e. day and night) and stocking densities according to GLMM. Light grey shapes indicate the dark period of the photoperiod. Different letters indicate significant statistical difference between groups (GLMM followed by Tukey HSD post-hoc test, $p < 0.05$).

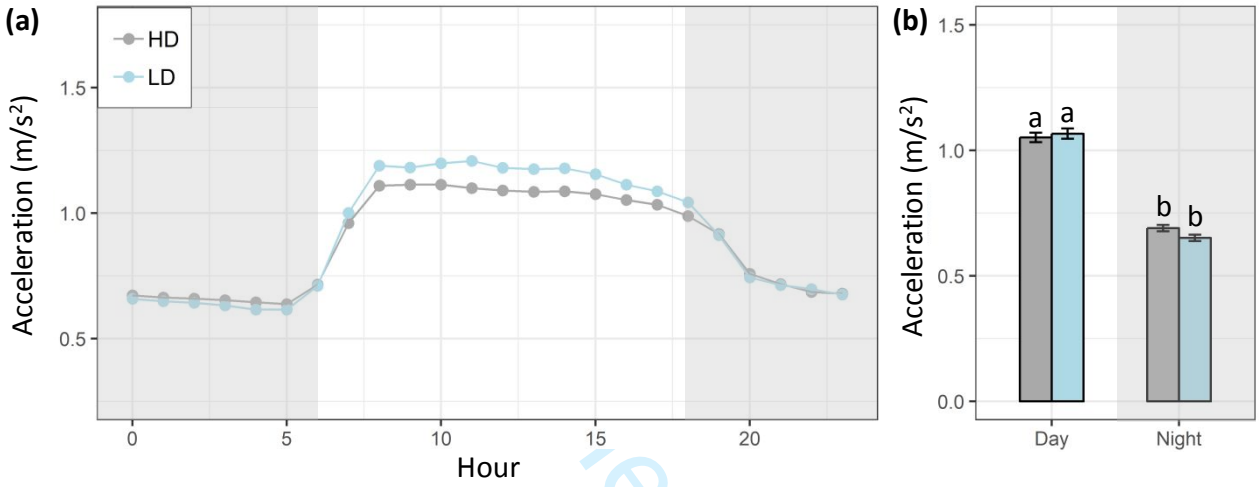

FIGURE 6. Physiological parameters of the blood at the start and the end of experiment for fish in high density (HD, grey; n=15) and low density (LD, blue; n=15). **(a)** Cortisol (ng/mL); **(b)** Glucose (mg/dL); **(c)** Lactate (mg/dL); **(d)** Hematocrit (HCT, %); **(e)** Red blood cell count (RBCC, 10^6 cells/mm^3) and **(f)** Lysozyme (µg/mL). The central line of the boxplot indicates the median and the boxes the quartiles, with the whiskers covering 95% of the values. Outliers are represented by points. Different letters indicate significant statistical difference between groups (ANOVA followed by Tukey HSD post-hoc test, $p < 0.05$).