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07 February 2025

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ABSTRACT

Salmo (trutta) marmoratus is an endemic specie in the North of Italy, subjected to hybridization with domesticated strains of trout. Native populations are managed by supportive release in the rivers. Wild breeders are captured, confined in facility for short periods and then released in the river after artificial fertilization. Premature mortality during confinement and post release mortality in river have been observed in breeders supporting the view that confinement stress could be the cause. Twenty-six adult individuals of trout were captured from a river by electrofishing and stocked in two tanks, the first one (RF) provided with artificial refuges to simulate the natural environment and covered by dark panels; the second tank (TR) was only partially covered by dark panels and without artificial refuges. All the other conditions were identical and animals were fed *ad libitum* with natural food collected in the same river. After 50 days, from a third group of 8 trout (WD) captured in the same river by a 5 minute electrofishing session, blood samples were sequentially collected for the assessment of serum cortisol response to serial repeated handlings. With the same sequential method, individuals of the RF and TR experimental groups were sampled. Cortisol levels were compared between groups by ANOVA. Biomass densities decreased during the experiment due to premature mortality of the largest individuals in both the RF (7.69%) and TR (30.77%) groups. At the end of the experiment, data clearly demonstrated that after a stressing confinement, the TR group shown a reduced poststress response to the successive serial handlings. Vice versa the group RF, that experienced a more careful confinement, responded to the second serial acute stressing manipulation in conformity as the group WD that was not confined. Cortisol data support the hypothesis of impaired cortisol response as a consequence of oversecretion due to uneasiness during the short-term confinement in artificial environment without refuges or due to "chronic" stress. Furthermore our data demonstrate that cortisolemia is not adequate as a marker for chronic stress but cortisol response to serial acute stress, in relation to time, can be used for the monitoring of stress level and welfare in trout breeders during confinement. The use of refuges and shadow in the artificial environment shown to play a relevant role in the survival of wild breeders of trout during confinement in the spawning season.

Key words: Confinement, Cortisol, *Salmo trutta*, Stress, Trout.

RIASSUNTO

EFFETTI DELLO STRESS DA MANTENIMENTO E MANEGGIAMENTO IN AMBIENTE ARTIFICIALE
SULLA TROTA SELVATICA IN PERIODO RIPRODUTTIVO

Salmo (trutta) marmoratus *è un endemismo dell'Italia settentrionale soggetto a progressiva ibridazione con trote di ceppo alloctono introdotte nei corsi d'acqua per la pesca sportiva. Le popolazioni selvatiche residue vengono recuperate e rinforzate mediante introduzione di stadi giovanili prodotti dalla feconda-zione artificiale di adulti prelevati dai fiumi. In attesa della maturazione gonadica i riproduttori vengono confinati in vasche dove a distanza di alcuni giorni si osserva una certa mortalità che è stata documen-tata anche in forma differita dopo il rilascio in ambiente naturale. Si ipotizzano cause derivate dallo stress accumulato durante il confinamento in ambiente artificiale. Da un corso d'acqua sono stati catturati mediante elettropesca un totale di 26 soggetti selvatici sessualmente maturi che sono stati equamente ripartiti in due vasche denominate RF ed RT, delle quali una (RF) quasi totalmente oscurata e provvista di tubi che simulano i rifugi utilizzati in natura da questi animali, mentre l'altra (RT) parzialmente scoper-ta e senza rifugi. Tutte le altre condizioni sono state mantenute identiche nei due gruppi. L'alimentazione era costituita da prede naturali prelevate nello stesso corso d'acqua. Trascorsi 50 giorni da un terzo grup-po (WD) costituito da 8 pesci pescati nello stesso ambiente e con le stesse modalità, è stato effettuato un campionamento sequenziale delle trote subito dopo la cattura, per la raccolta dei campioni di sangue che sono stati utilizzati per la quantificazione del cortisolo nel siero. Con lo stesso metodo, sono state quindi campionate le trote dei 2 gruppi sperimentali TR e RF. I progressivi livelli di cortisolemia raggiun-ti dalle singole trote campionate sono stati analizzati per gruppi, mediante test ANOVA. Rispetto agli indi-vidui dei gruppi RF e WD, quelli del gruppo TR hanno mostrato una limitata capacità di rispondere alla manipolazione sequenziale con un ridotto incremento della cortisolemia nel tempo. L'incremento indivi-duale di cortisolemia in seguito al campionamento sequenziale non ha invece mostrato differenze signi-ficative tra i gruppi RF e WD. La mortalità che ha interessato prevalentemente gli individui di taglia più grande, è stata superiore nel gruppo TR rispetto ad RF. Si considera verosimile una ridotta secrezione di cortisolo come reazione di adattamento conseguente ad una continua ipersecrezione imputabile a stress di tipo "cronico", indotto dalla inadeguatezza delle condizioni artificiali di confinamento in TR. I dati otte-nuti contribuiscono a provare l'inadeguatezza dei soli valori del cortisolo ematico come indicatore di stress cronico, che potrebbe invece essere evidenziato studiando il variare della cortisolemia nel tempo in rispo-sta a stress seriali di tipo acuto. Si dimostra inoltre che l'oscuramento e l'utilizzo di rifugi all'interno delle vasche artificiali sono accorgimenti in grado di migliorarne le condizioni di confinamento e di ridurre la mortalità negli adulti tenuti in cattività durante il periodo riproduttivo.*

Parole chiave: *Cattività, Cortisolo,* Salmo trutta, *Stress, Trota.*

Introduction

The conservation and restocking of endemic freshwater species is a relevant topic in Italy. The marble trout *Salmo (trutta) marmoratus* (Cuvier, 1817) is included in the list of the endangered species because it can hybridize with domesticated strains of *Salmo (trutta) trutta* introduced in freshwaters for fishing. Native populations are managed by restoration projects and supportive release in the rivers of fry fish and young trout. Wild breeders are captured during the spawning season, confined in facility or hatchery tanks for a short period and released in the river after artificial fertiliza-tion. Following this conservation policy, hybridization with the brown trout is reduced and the survival rate from egg to the fry stage can be greatly increased as compared to that occurring in the natural environment. In our fieldwork and fishery experience, wild trout show higher sensitiv-ity to capture, handling, transportation and confinement than domesticated strains. This is largely documented by the high post handling and post captivity premature mor-tality in hatcheries. Repeated handlings,

stripping and confinement chronic stress in breeders, could be also the cause of the documented postrelease mortality in river. The physiological effects of the stress have been investigated in domesticated salmonid fish (Barton, 2000) and very little in wild trout (Woodward and Strange, 1987), but the physiological responses to chronic stress in adults of wild trout during the spawning season were never investigated. For the success of the threatened species conservation programs, in captive breeding aquaculture is crucial to define appropriate fieldwork and hatchery procedures in order to minimize post release mortality of breeders as a consequence of handling, stripping and confinement stress. The effect of stress in fish farming has been widely investigated in many aquaculture species in the last decade (Iwama *et al.*, 1995, 2004; Ron *et al.*, 1995; Wagner *et al.*, 1995; Klinger *et al.*, 1996; Barton and Dwyer, 1997; Barton *et al.*, 1998; Watson *et al.*, 1998; Forsberg *et al.*, 1999, 2001; Barton, 2002; Peres *et al.*, 2004; Ribas *et al.*, 2004). Cortisol is a primary stress hormone in teleosts and the effects of high hormonal levels can be severe, leading to decreased disease resistance through immunosuppression, reduced growth and reduced reproduction (Anderson, 1990; Wendelaar Bonga, 1997). Plasma cortisol stress response to various traumatic procedures such as transportation, anesthesia, thermal shock, handling and confinement, have been investigated in many species (Robertson *et al.*, 1988; Barton and Iwama, 1991; Tort *et al.*, 2002; Haukenes and Barton, 2004; Barton *et al.*, 2005; Quigley and Hinch, 2006) and even in trout (Barton and Peter, 1982; Pickering and Pottinger, 1989; Barton, 2000; Sicuro *et al.*, 2005). The increase of blood cortisol concentration is the most widely used indicator of acute stress in response to a wide variety of adverse stimuli in trout. After exposure to a stressor, cortisol level increases and generally returns to basal level within few hours (Barton, 2000), whereas in presence of repeated stimuli that lead to chronic stress, blood cortisol tends to increase and stabilize later to high levels. In these conditions, a subsequent reduced interrenal responsiveness of the hypothalamic–pituitary–interrenal (HPI) axis due to oversecretion can be observed (Haukenes and Barton, 2004). In the wild, adult of marble trout spend most of the time in hiding places moving out from underwater refuges just for feeding. During confinement in the common hatchery tanks, we suspect that breeders are forced to interact each other in the artificial environment and these conditions could lead to a "chronic" stress in pre-spawning period. Our research group is investigating no-invasive monitoring tools of stress levels in wild trout captive breeding. The present experiment was designed to preliminary investigate the cortisol response of trout to a second stressing manipulation, following confinement stress under two different environmental conditions and to evaluate the use of impaired poststress cortisol responses as a tool for the monitoring of the wellness of breeders during short-term captivity.

Material and methods

Wild trout were captured by soft electrofishing from a water channel of the Piave basin (Selva del Montello, Province of Treviso, N/E of Italy) in December 2005 during the spawning season. After three minutes of travel in oxygenated water tanks, a total of 26 adults of wild trout were carefully stocked in two identical rectangular fibreglass tanks (L=3.0 m., W=0.9 m., H=0.6 m.) both provided with a constant filtered water flow of 20-25 l/min, withdrew directly from the same water channel. During the experiment, water temperature

and dissolved oxygen were very constant, ranging from 5°C to 6°C and from 11.3 to 11.9 mg/l respectively. Fish weight were annotated after the capture and at the end of the experiment before blood sampling, dissection and sex determination (Table 1). The first group of trout (TR) was confined in the tank covered with dark panels for the 75% of the water tank surface. The second tank (RF) was covered for the 93% of the water tank surface and provided with 14 plastic tubes (diameter =200 mm, length =350 mm) placed on the bottom by stones. Every three days at the sunset, fishes of both the tanks were fed *ad libitum* with natural aquatic organisms, like worms, larvae and small fish directly collected from the water channel; almost all the trout showed a partially filled stomach at the end of the experiment. Fish conditions were daily checked in the dark after the sunset by an infrared camera to minimize disturbance. Eight days after the capture, both tanks were gently handled in the dark to clean out foul matter from the bottom, caused by a little cloudy water after four days of rain. The experiment was carried on paying attention that human presence and noises would not affect fish behavior. Fish were never handled for the next 49 days till the end of the experiment in the self-cleaning tanks. After 57 days, respecting the interval of 15 minutes between two subsequent captures of fishes in the tank, blood samples were collected from each survived individual after instantaneous neck dislocation. This sampling plan simulates the common manual inspection of the gonad maturation, stripping of eggs and milt of the wild breeders in the hatchery. The sample of blood was collected with a syringe from incised gills and immediately centrifuged at the environmental temperature of 6°C to separate the serum. A lower quantity of blood was collected from the trout named 01RF due unexpected coagulation, so that, statistical analyses were prudentially performed excluding and later including the 01RF cortisol data. Serum samples were kept in ice during collection and then stored at -22°C after three hours. In the same water channel a third group of eight trouts (WD) similar in body size to fish of the experimental group were captured in a 5 minutes of rapid electrofishing session. The eight fishes were quickly transported to the valley-hatchery and the blood sampling session started 15 minutes after the capture of the first fish from the channel. The WD group was simply utilized as a "blank" to assess the cortisol response of trout immediately after the capture, without confinement experiences. Photos of the lateral body side of each trout were made immediately after neck dislocation and the exact time was recorded on the digital memory of the photo camera. Serum cortisol levels were assessed using a RIA kit (DSL-2000, Alifax, Italy).

Data analysis

Pearson correlation coefficient has been used to test the correlation between serum cortisol concentration and body weight. Correlation tests between cortisol levels and the "time of samplings" were performed in order to assess the magnitude of poststress responses within each experimental group. "Time of samplings" refers to the intervals of time between the first and subsequent individuals sampled for each group. For the survived fish of TR and RF groups, within group and among groups ANOVA were applied on body weights, cortisol levels and mortality data, to test for difference between the two sub-datasets of males and females. Best-fit values and goodness-of-fit quantified by sum-of-squares were used on cortisol data / "time of samplings", to compare linear, logarith-

Table 1.   Summary of data collected and computed during the experiment.

| group TR (traditional tank) | | | | | group RF (tank with refuges) | | | | | WD (wild trout from the river) | | | | |
N	W	S	C	sex°	N	W	S	C	sex°	N	W	S	C	sex°
01 TR	215	-	12.94	m	01 RF	434		5.20	m	01 CL	335		52.76	m
02 TR	340		15.30	f	02 RF	230	-	16.00	f	02 CL	290	-	31.16	f
03 TR	180	-	38.40	m	03 RF	502		35.77	m	03 CL	433		70.91	f
04 TR	162	-	25.97	f	04 RF	688		44.98	f	04 CL	327		102.40	m
05 TR	390		46.69	f	05 RF	294	-	41.45	f	05 CL	247	-	57.50	f
06 TR	322	-	53.66	f	06 RF	664		137.4	m	06 CL	251	-	234.20	f
07 TR	164	-	50.79	m	07 RF	204	-	74.84	m	07 CL	289	-	102.60	m
08 TR	458		64.43	m	08 RF	208	-	69.53	m	08 CL	234	-	66.10	m
09 TR	160	-	44.95	f	09 RF	262	-	100.93	f					
10 TR	410		/	m d	10 RF	390		144.3	m					
11 TR	465		/	m d	11 RF	184	-	154.5	f					
12 TR	545		/	f d	12 RF	130	-	89.00	m					
13 TR	310	-	/	f d	13 RF	340		/	f d					
a	317	-	39.24			348		76.16	82,60(*)		301	-	89.70	
b	266	-				349					=		=	
c	433					340					=		=	
	3928					4219			initial biomass for each group (g)					
	3816					4194			final biomass (theoretical) for each group (g)					
	4242					4557			initial biomass density for each group (g/m³)					
	4121					4530			final biomass density (theoretical) for each group (g/m³)					
	2214					3880			biomass of the survived fish at the end of the experiment (g)					
	2391					4190			real biomass density at the end of the experiment (g/m³)					

In columns N, W, S, C are reported: sample Name, body Weight, Size (-) and Cortisol levels at the end of the experiment.
Row a: average values. (*)= average value excluding sample 01RF (see text);
Row b: mean body weight of survived trout;
Row c: mean body weight of trout died before the end of the experiment;
-= below the overall mean body weight (34 samples; mean body weight = 325.2 g).
°= m = male; f = female: d = dead before the end of experiment.

mic, inverse, quadratic, cubic, power, S, exponential and logistic models, in order to investigate the pattern of poststress cortisol responses in all the experimental groups. Test for normal data distribution and homogeneity of variance (Hartley, Cochran C and Bartlett K-sq) have been performed before the one-way ANOVA and Post Hoc comparisons on cortisol data. SPSS and STATISTICA software were used for statistical analyses; significance was accepted at $P<0.05$.

Results

Daily inspections and observations by infrared night vision micro camera revealed that all the trouts of the RF group spent most of the time into, or very close, the underwater artificial refuges. By contrast, most of the time, trout of the TR group stayed close to each other in the darkest part of the tank. Biomass densities decreased during the experiment because of premature mortality in the RF (7.69%) and TR (30.77%) groups.

ANOVA test for significance between the initial and final body weights of fish showed that during the experiment there were not significant variations in the body weight of survived trout, in both the TR and RF groups (P>0.05). Altogether, the loss of biomass during the experiment was 2.85% in the TR group (3928 g initial biomass / 3816 g final theoretical biomass) and 0.59% in the RF group (4212 g initial biomass / 4194 g final theoretical biomass). In both the TR and RF groups, ANOVA tests on body weight, cortisol levels and

Figure 1.   Wild trout held in tank without refuges (TR group).
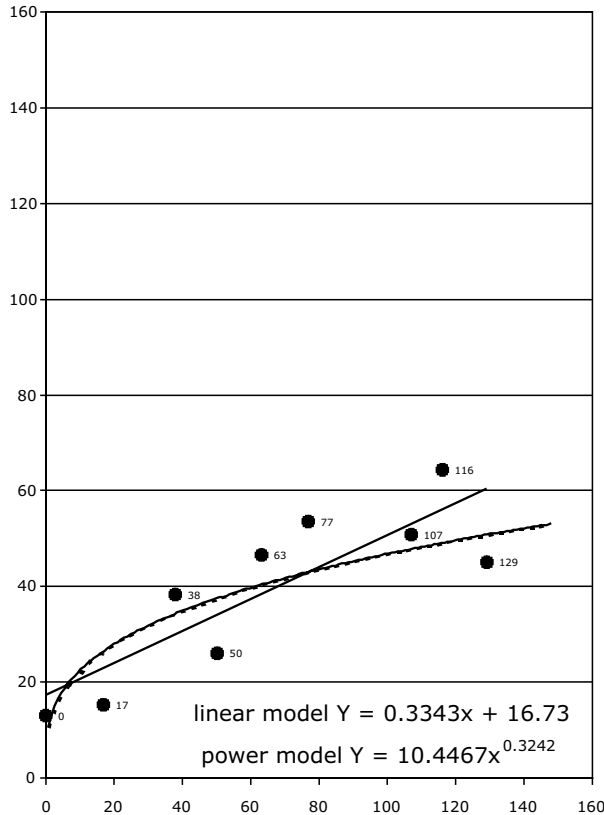
linear model Y = 0.3343x + 16.73

power model $Y = 10.4467x^{0.3242}$

Y axis = Serum cortisol levels (ng/ml);
X axis = Within group sequence of blood collection ("time of samplings", see text).
See also note to Figure 2.

mortality data, excluded differences in size of fish, cortisol responses and premature mortality, related to the sex of individuals (P>0.05). At 11, 20, 23 and 27 days from the beginning of the experiment, four trout of the TR group died (mean body weight =432.5 g). Only one fish (body weight =340 g) of the RF group died one day before the end of the experiment. *Post mortem* examination excluded pathological inputs in mor-

tality. In four of the five died trouts, the body weights were higher than the overall average body size (26 trout; average body weight = 332.7 g). In all the three groups, the correlation between serum cortisol level and body weight was not significant (for the TR group r=0.36 and p=0.34; for the RF group r=-0.199 and p=0.95; for the WD group r=-0.238 and p=0.57). Test for normal data distribution and homogeneity

Figure 2.   Wild trout held in tank provided with refuges (RF group).
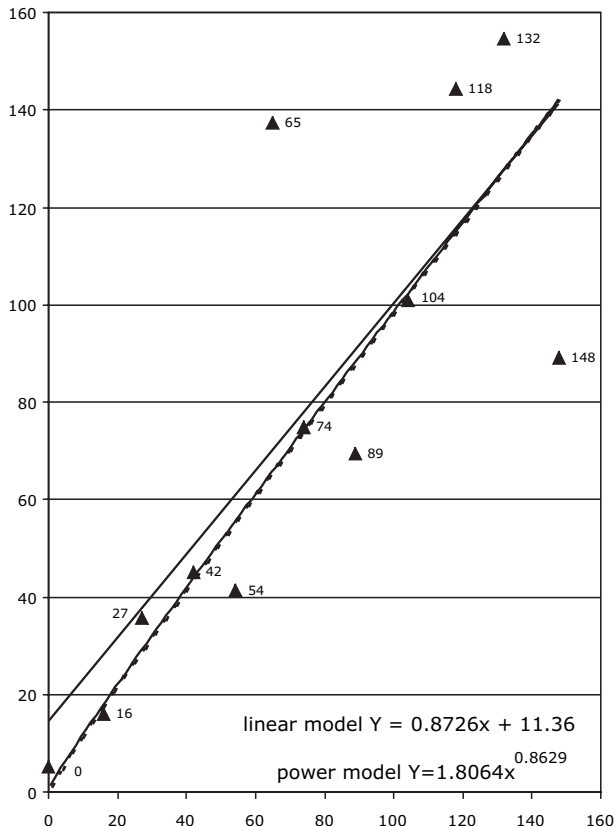
linear model Y = 0.8726x + 11.36

power model Y=1.8064x$^{0.8629}$

Y axis = Serum cortisol levels (ng/ml);
X axis = Within group sequence of blood collection ("time of samplings", see text).
The slope of the linear model and the power model curves are different in the two experimental groups (TR in Figure 1 and RF in Figure 2). In relation to time, serum cortisol postacute stress response resulted from 2.5 to 2.9 times higher in the RF group then in the TR group (Figure 1).

Table 2.   ANOVA: P values for Duncan and LSD post-hoc test on serum cortisol values.

Group	TR	RF	WD
Average ng/ml	39.236	82.609	89.703
RF (Duncan P)	0.0541	-	
WD (Duncan P)	0.0340*	0.7437	-
RF (LSD P)	0.0459*	-	
WD (LSD P)	0.0327*	0.7424	-

Second row: average cortisol values for each group of trout.
* = significant probability values (P<0.05).

of variance assured the correct use of ANOVA for cortisol data (Hartley F-max = 12.75; Cochran C=0.61; Bartlett K-squared = 9.71; d.f.=2; p=0.0078). One-way ANOVA and Post Hoc outcomes (Table 2: LSD tests) shown significant p values in TR group when compared with both the RF and WD group. Marginally lack of significance at the Post Hoc test was found comparing the TR and the RF groups with the sample 01RF included in the dataset. The TR group showed the lowest serum cortisol levels (mean value = 39.24 ng/ml) whereas the highest levels were found in the WD group of wild trout never confined in the hatchery (mean value = 89.70 ng/ml). High significant correlations between serum cortisol responses and the "time of samplings" were found in both experimental groups (Figures 1 and 2) with the following outcomes: for the TR group, n=9; Pearson r=0.847; p(2-tailes) =0.0046. For the RF group, n=11; Pearson r=0.749; p(2-tailes) =0.0089. The lowest cortisol levels in both experimental groups were found in the first samples collected during the sampling session, but the linear regression trendlines for the two groups were different in slope, with equation y=0.3343x+16.729 in the TR group and y=0.8356x+15.152 in the RF group (y=0.8726x+11.365 in the RF group including the sample 01RF). In the WD group correlation was not significant (r=0.413, p=(2-tailes)=0.3093). In both the TR and RF groups, best-fit values and goodness-of-fit comparison on cortisol-data / "time of samplings" (Figure 1 and 2), indicated that the power model equation has the lowest sum-of-squares and the lowest significant p values at the F-test, with the follow outcomes:

- TR group: Rsq=0.745; d.f.=7; F=20.43; *p=0.0027;

TR-equation: Cortisol=10.44672x(minutes^0.32424);

- RF group (11 samples): Rsq=0.797; d.f.=9; F=35.06; *p=0.0002;

RF11-equation: Cortisol=1.28924x(minutes^0.94048);

- RF group (12 samples including RF01): Rsq=0.905; d.f.=10; F=95.65; *p =0.0000;

RF12-equation: Cortisol=1.80640x(minutes^0.86295).

The ratio between the slope of RF and TR curves was 2.50 (0.8356/0.3343) for the linear model and 2.90 (0.94048/0.32424) for the power model.

Basal serum cortisol levels, estimated by extrapolation at the time zero, were lower in the RF group than in the TR group: 11.36 ng/ml in the RF group and

16.73 ng/ml in the TR group in accordance with the linear model; 1.81 ng/ml in the RF group and 10.45 ng/ml in the TR group in accordance with the power model.

Discussion

Biomass densities were very low in these experimental groups respect to the ordinary biomass densities in fish farms. In absence of pathologies, we suppose that premature mortality of the largest wild trout was a consequence of constriction or uneasiness during the short-term captivity in tank. Considering that wild fish started to die after the 11th day and the highest mortality was between the 20th and the 27th day of experiment, we suspect a relation between premature mortality and harmful effects of prolonged environmental stress in the TR group. Aggressive social interaction should also considered but usually, younger individuals are mainly affected by aggressive interaction in heterogeneous groups of wild trout held together and in our experience premature mortality also affect breeders of trout held in tanks alone, in total absence of social interactions. Our result clearly indicates that during the spawning season, the harmful effects of confinement in artificial environment mainly affect the largest individuals. Biomass density and number of individuals in the TR group, decreased during the experiment due to premature mortality, resulting lower than biomass density and number of individuals respect to the RF group. Basing on these evidences, in artificial environment provided with refuges and shadow, breeders of trout seem to better tolerate overdensity and confinement. Survived trout were the smallest in size but we did not find any significant correlation between cortisol levels and body weights. According to these evidences, the

reduced postacute stress cortisol response in serum of the TR group should be attributed to inadequacy of the artificial environment, proved by the loss of body weight in the survived trout during the experiment but also by the highest premature mortality as an ultimate response to environmental stress. The reduced serum cortisol responses could also be explained assuming a reduced interrenal responsiveness as a consequence of prolonged stress in the TR group; therefore, inhibition or exhaustion of the HPI axis due to oversecretion and the General Adaptation Syndrome (GAS) should be considered (Barton, 1997; Ford et al., 1997; Henzen et al., 2000; Rotllant et al., 2000; Bugajski et al., 2001). A reduced corticosteroids response following an acute stressor has been mimicked by prior continuous treatment with cortisol-impregnated feed, demonstrating the negative-feedback effect of elevated circulating cortisol on the HPI axis (Barton et al., 1987; Rotllant et al., 2000). Cortisol has been shown to induce self-suppression by negative-feedback of its secretion directly at different levels: (i) the interrenal tissue (Bradford et al., 1992); (ii) the ventrodorsal hypothalamus, suppressing the synthesis and/or release of CRH (Fryer and Peter, 1977); and/or (iii) the pituitary, inhibiting ACTH release (Fryer et al., 1984). Thus, long-loop and ultra-short-loop feedback regulatory mechanisms have been clearly documented in fish. In the present experiment, adaptation should be occurred in the TR group: trout subjected to environmental uneasiness during the short-term captivity, showed a trend to levels below values of the WD and RF groups, adjusting or compensating for the disturbance to regain homeostasis. This behave correspond to the second step of the GAS. The cortisolemia clearly demonstrated that after a more stressing confinement, the TR

group shown a reduced response to the successive stressing manipulation, consisting of a sequential fish sampling for the blood collection. Vice versa the group RF, that experienced a more careful confinement, responded to the second serial acute stressing manipulation in conformity as the group WD that was not confined (Table 2). In the TR group, the hypothesis of impaired stress response likely due to environmental stress (Arends *et al.*, 1999; Quigley and Hinch, 2006) is supported by the difference between the linear and the power curves of cortisol levels / "time of samplings". The "time of samplings" could also be considered as "the number of repeated acute stress" because during the final sampling session fishes were took out from the tanks one at time and the repeated handlings should have represented a repeated acute stress in individuals sampled later. For example, the last individual sampled in the TR group was subjected to disturbance of capture 8 times in 2 hours, enough time to progressively increase the serum cortisol in response to serial acute stimuli. Basal levels of cortisol for unstressed salmonid fish are normally below 10 ng/ml (Gamperl *et al.*, 1994; Wendelaar Bonga, 1997). The increase of serum cortisol in all the experimental groups in relation to the "time of samplings" indicates a progressive stress response in fish caused by repeated handlings. These levels are similar to those found in rainbow trout subjected to confinement stress (Pottinger and Moran, 1993; Ruane *et al.*, 1999) and in salmonid fish subjected to handling and transport stress (Barton, 2000). In the RF group of our experiment, the curve computed with the power model was very similar to the curve computed with the linear model (Figure 1). On the contrary, power and linear curves computed on cortisol responses of the TR group were very different. Furthermore,

serum cortisol values extrapolated at the time zero were higher in the TR group than in the RF group, suggesting that in trout of the TR group the basal cortisol levels were high during confinement. These evidences support the hypothesis that low postacute stress cortisol response in the TR group was a reduced trout's normal capacity to respond to serial acute stimuli likely due to exhaustion for chronic oversecretion during confinement in a tank without shadow and any hiding protection. Furthermore, our outcomes support the view that the degree of response depends on both the severity and duration of the stressor (Barton *et al.*, 1980; Barton and Iwama, 1991). High correlation between acute poststress cortisol responses and the "time of samplings" (or "the number of serial acute stimuli") were found, but differences in cortisol levels, trendline slopes and trendline equations (Figures 1 and 2) clearly demonstrated a different magnitudes of responses in the two experimental groups. We hypothesize that the magnitude of response but also the difference between the linear and the power model equations of poststress cortisol response could be used as tools for the monitoring of environmental stress in medium and short term breeders captivity. For example, the RF power equation could be assumed as a reference for cortisol response in unstressed wild trout during captivity; it should be possible to use the first interception point between the RF and a target group curves as an indicator of HPI axis suppression. In our experiment, the interception point between RF and TR power equations was at 30 minutes, but in target groups affected by lower degrees of environmental stress, we expect lower HPI axis suppressions and interception points >30 minutes. Following this approach, would be necessary to define a precise equation for cortisol response in healthy

and unstressed wild trout during confinement in artificial environment (true control group), but it is impossible because confinement is a stressor by itself for wild animals. Alternatively, the linear mode equation could be assumed as the theoretical response in absence of HPI axis suppression, whereas the first interception point between the linear and power equations could be used as indicator of HPI axis exhaustion due to oversecretion in presence of stress. In the TR group of our example, the power curve rapidly approximates to the linear curve and the interception point corresponds to 6 minutes, whereas in the RF group the interception between the two equations corresponds to 108 minutes. In the TR group the highest premature mortality was observed and the magnitude of the suppressed poststress cortisol response was 2.5÷2.9 times lower respect to the other groups. Furthermore, ANOVA Post Hoc on cortisol levels was not significant between the RF group and the WD group. In light of all these evidences, we conclude that in short-term confinement, the artificial environment condition plays a relevant role in the survival and welfare of wild trout. The presence of refuges and shadow make the artificial environment much more similar to the wild environment, efficiently reducing social disturbances, territorial competition and the negative effect of chronic stress and constriction, likely simulating a lowest biomass density. Wild individuals of both TR and RF experimental groups were kept under controlled conditions for a couple of months, experiencing similar early life histories during confinement. On the contrary, trout captured directly from the river, were not under control and each individual could have experienced in the wild a different early life history as hypothesized by Woodward and Strange (1987), Barton

(2000). For this reason one could not expect homogeneous poststress cortisol response in the WD group; nevertheless, the lack of correlation between cortisol levels and "time of samplings" could also be due to the fact that blood sampling did not respect the exact order of capture by electrofishing. However, cortisol response of the WD group was essential in assessing the conserved normal capability of the RF group to react in response to acute stressor. The low-density confinement was necessary because wild trout are much more sensitive to constriction than domesticated trout; therefore, the number of samples in our experiment was adequate for a first exploration of a novel approach for the monitoring of confinement stress in wild trout but not adequate for the accurate calculation of equations.

Conclusions

Fishing restrictions for the conservation of endemic freshwater species gave no means to use a higher number of fish in this experiment to discriminate effects due to uncontrolled tank-to-tank differences from the genuine effects of artificial refuges in the tank (within-treatment replication). However the results of this study suggest that in wild trout confinement during the spawning season, the magnitude and the pattern of poststress cortisol response after serial acute stimuli could be used as tool for the monitoring of middle and short-term environmental stress. The physiological responses to acute stressor and a method for the assessment of confinement stress in wild breeders of trout during the spawning season were never investigated before, but similar results were obtained by Barton and colleagues (2005) in juvenile domesticated gilthead sea bream, using many indicators for the assessment of fish physiological

response. This suggest that further investigations are necessary to get deeper insight this topic in wild species, better combining the use of cortisol response with other indicators, as suggested by Barton (2000). Environmental conditions in captive breeding play a relevant role in welfare and survival of wild trout during confinement. In large wild trout, the presence of artificial refuges and shadow in the tank, reduce mortality and probably the stress caused by constriction, overdensity, visual interaction and reciprocal disturbances. These are focal points in captive breeding aquaculture concerning the conservation and restoration of the endemic trout *Salmo (trutta) marmoratus* in Italy, because the small wild populations are threatened by genetic introgression with domesticated strains of trout. Adult individuals of marble trout are captured by electrofishing at the beginning of the spawning season, held in hatchery tanks until November/December and released in rivers after artificial fertilization. The monitoring of stress is necessary to define appropriate procedures of capturing, transporting, stocking, feeding and handling in fieldwork but also to define appropriate methods for stripping and for a no-invasive assessment of fish physiological conditions in the hatchery, in order to minimize mortality during captivity and post release mortality of breeders in the rivers.

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