The physiology of rainbow trout in social hierarchies: two ways of looking at the same data

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Abstract Salmonids form dominance hierarchies in environments, where space or food are limiting. Our first objective was to investigate the physiology of individual rainbow trout in 4-fish hierarchies. Our second was to compare conclusions drawn from grouping physiological data on the basis of social rank with those based on relating individual physiology to individual aggressive behavior. To create a social hierarchy, groups of 4 juvenile trout were fed (1 % ration) using a darkened feeding container, twice daily (morning and evening). Each morning feeding was videotaped to record aggressive behavior, thereby facilitating the assignment of a social status rank to each fish. On days 5 and 10–11, physiological parameters were measured in fish fasted for 24 h. Social hierarchies formed in all tested groups. One fish would become dominant, whereas the three subordinate individuals would each assume a stable social rank. When classified according to this social rank, the three subordinate individuals all displayed similar physiology, different from the physiology of the dominant fish. The latter included higher ammonia excretion rate, greater protein utilization in aerobic metabolism, greater feeding, higher specific growth rate, greater increase in condition factor, and lower routine oxygen consumption rate. However, when individual aggression was taken into account, a continuous gradient was observed between aggression and physiology for most parameters, regardless of social status. These relationships could be improved by normalizing the aggression score to the overall level of aggression in each hierarchy. We argue that individual behavior should be considered instead of just social rank when studying the physiology of trout in social hierarchies.

Keywords Aggression · Social rank · Feeding · Growth · Oxygen consumption · Ammonia excretion

Introduction

Salmonids form social hierarchies in settings with limiting resources such as food and space (Chapman 1966). Establishment of this 'pecking order' is accomplished through agonistic behavior, where fish use aggression to compete for the limiting resource. Stable hierarchies are thought to be beneficial to both subordinate and dominant individuals by reducing aggressive behavior as compared to unstable hierarchies (Gurney and Nisbet 1979). Dominant individuals are often viewed as the 'winners' in the hierarchy, displaying higher growth rates, higher food consumption and having greater access to mates (Pottinger and Pickering 1992; McCarthy et al. 1992). Subordinates are seen as the 'losers', exhibiting physical damage, lowered immunity, slower growth rates (McCarthy et al. 1992; Abbot and Dill 1989; Peters et al. 1988), and elevated plasma cortisol levels (Pottinger and Pickering 1992; Sloman et al. 2000b, 2001; Hoglund et al. 2002). Higher metabolic rate also often characterizes subordinates (Sloman et al. 2000c). The unequal feeding which occurs could lead to differential ammonia excretion rates (Alsop and Wood 1997; Bucking and Wood 2008), but this parameter has not been assessed previously within a social hierarchy.

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It has become common practice to average data collected from fish of equal social status from different hierarchies (e.g. Abbot and Dill 1989; Sloman et al. 2000b, c, 2001; Harwood et al. 2003). This approach is informative because it highlights physiological differences among fish of different social statuses that exist within a hierarchy. However, possible individual physiological and behavioral correlations within social hierarchies will be masked when this approach is applied. By "binning" statuses, we lose potential information about variation among hierarchies in the behavioral dynamic and associated variation in physiology. Other studies have correlated individual behavior with individual physiology in paired contests. For example, in convict cichlid fish (*Archocentrus nigrofasciatum*), higher submissive behavior correlated with higher bile mass (Earley et al. 2003). A positive correlation was observed between muscle lactate concentrations and contest dynamics in the same species (Copeland et al. 2011). In rainbow trout, a positive correlation was seen between fight duration and the neutransmitters serotonin and noradrenaline (Øverli et al. 1999) as well as a negative relationship between confinement-induced plasma cortisol concentrations and aggression scores (Øverli et al. 2004). However, comparable investigations in social hierarchies of more than two individuals are rare. The work of Sloman et al. (2008), who related individual behavioral scores to higher plasma cortisol levels and higher growth rates in 12-trout hierarchies, is one such example.

Our first objective was to use non-invasive methods (behavioral observations, individual respirometry, growth, feeding), as well as cortisol measurements to investigate how social status affects the physiology of individual trout in a 4-fish dominance hierarchy. Ammonia excretion rates, which have not been recorded in the previous hierarchy studies, were of special interest because when measured together with oxygen consumption, they indicate the degree to which proteins and amino acids are used to fuel aerobic metabolism (van den Thillart 1986). Our specific hypotheses were (1) that each social rank would display a distinct physiological profile for most of the traditional parameters, different from the other ranks; and (2) that dominant fish would rely less on protein and amino acid metabolism, so as to direct more nitrogen into protein growth. The null hypotheses were that neither of these phenomena would occur. The results were surprising, inasmuch as neither of the specific hypotheses were supported. Our finding that all subordinate ranks displayed a similar physiological profile forced us to re-assess the approach commonly used for analyzing physiological data in dominance hierarchy studies. Our second goal therefore was to compare conclusions drawn from grouping physiological data on the basis of social rank with those based on the relating individual physiology to individual aggressive behavior.

Methods and materials

Procedures were approved by the McMaster University Animal Care Committee and complied with the regulations of the Canada Council for Animal Care.

Experimental animals and holding conditions

Juvenile rainbow trout (6–10 g) were purchased from Humber Springs Trout Hatchery (Orangeville, Ontario), and held in batches of 50 fish per 200-L aerated aquarium, supplied with dechlorinated Hamilton tap water (12 °C), pH $\sim$ 7.5, flow rate $\sim$1 L min^{-1}, photoperiod of 12.5 h light: 11.5 h dark, at McMaster University for 3 weeks prior to experimentation. Fish were fed a 1 % total tank weight ration with Martin's commercial dried pellet feed (1 point; Martin Mills Inc., Elmira, Ontario) three times per week. Water composition was: (in mmol L^{-1}) Na$^+$ = 0.5, Cl$^-$ = 0.7, Ca = 1.0, hardness $\sim$140 ppm as CaCO$_3$. The fish had sufficient space and access to food during holding conditions so that aggression was minimal and social hierarchies did not form.

Hierarchy preparation

Fish were anaesthetized individually in neutralized MS-222 (0.08 g tricaine methanesulfonate L^{-1}), weighed (0.01 g), and fork length (0.1 cm) was measured. Each fish was uniquely freeze branded to allow for visual identification. This was achieved using a surgical probe dipped into liquid nitrogen: the cold tip was pressed behind the head to form a distinctive mark. Fish were air-exposed for no more than 1 min and regained normal behavior after one day, with feeding occurring 2 days after anaesthetization. No severe side-effects were observed. Four sized-matched fish were then placed inside an aerated 30-L tank (53 × 26.7 × 30 cm) supplied with flowing water (water quality the same as holding conditions, though in-tank measurements of pH were routinely 7.2–7.4). A clear lid facilitated behavioral observations. Five pieces of PVC pipe (1 floating) (7 × 2.5 cm) were also added to the tank to serve as shelter.

Hierarchy creation and feeding regime

Data were obtained from a total of 7 separate hierarchies, each consisting of 4 trout, and each set up in an identical fashion in a 30-L tank, as outlined in "Hierarchy preparation". To create social hierarchies and record physiological differences between fish, a new technique was designed, using a darkened feeding container that could be closed as a respirometer. Fish were fed using a darkened, plastic container (17.8 × 14 × 12 cm, volume = $\sim$2.8-L)

Fig. 1 a, b Diagram of darkened feeding container and 'dummy' feeding container used for respirometry (17.8 cm length × 14 cm height × 12 cm width, volume = 2.8-L). After the feeding container was placed inside the tank, food pellets were deposited into the feeding container through the feeding tube, where the food was localized inside. To capture fish, a 'dummy' feeding container was placed inside the tank and as soon as a fish entered, the hinged lid was closed (**b**), confining the fish inside. The container was then used as a respirometer
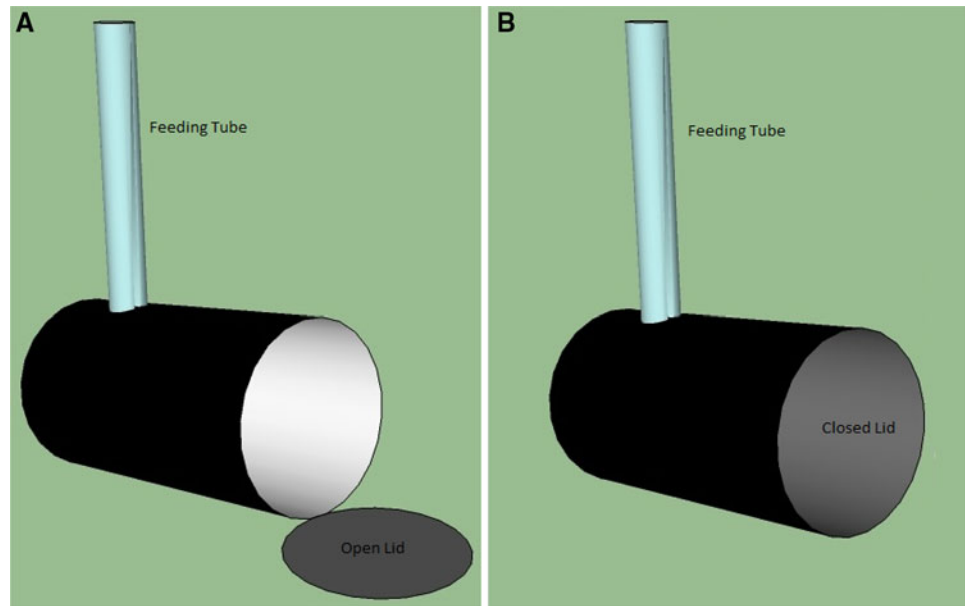
with a feeding tube attached to it, so that the food pellets were deposited inside the container. Food was, therefore, highly localized in one zone inside the darkened container. Fish associated the darkened feeding container with food and attempted to monopolize the feeding area (Fig. 1a).

Using this method, fish were placed on a strict feeding regime, consisting of two feedings daily of 1 % total tank biomass (morning between 7:30 and 9:00 AM and evening between 6:30 and 8:00 PM). Food was delivered pellet by pellet into the feeding tube, after which a small amount of tank water (collected with a small beaker) was used to flush the left-over pellets into the feeding container. Dispensing food pellets took less than a minute. The feeding container was left inside the tank for 15 min (during which a video camera recorded group behavior) for 10 consecutive days. The first 5 days are termed as period 1, with days 6–10 referred to as period 2. Data were obtained from a total of 7 separate hierarchies, all set up in an identical fashion.

Behavioral measurements

Morning feedings were selected as the behavioral study period because preliminary results showed higher aggression during the morning compared to evening feedings. During morning feedings, a video camera (Sanyo VPC-WH1, Osaka, Japan) was set up on scaffolding surrounding the tank to videotape aggressive behavior for 15 min which began at the start of feeding. Only behavior outside the feeding container could be recorded since the feeding container was dark. Individual feeding could not be determined from video data. However, to observe activity inside the feeding container during a feeding, a clear container (exactly the same as the darkened feeding container except the coloration had been removed) was used to feed two different established, stable hierarchies, each for a single feeding. These data were taken for observational purposes only, and were not included in the analyses. In addition, in preliminary experiments on two stable hierarchies, aggressive behavior was also monitored during a non-feeding period in the afternoon. Aggressive behavior was similar in both quantity and intensity between morning feedings (feeding container present) and afternoon periods when the feeding container was absent, so only data from the morning feeding periods were collected in subsequent trials. Notably, the level of afternoon aggression was also similar as compared to that later observed during morning feedings in other hierarchies.

Each aggressive act (chase or approach which caused the other fish to react) was scored 1 point, allowing for a dominance hierarchy to be recorded, with dominant individuals having higher scores than subordinate individuals. Therefore, rank 1 fish were the most dominant and rank 4 were the least dominant.

Physiological measurements

Fish were starved for 24 h prior to physiological measurements, which were recorded at the ends of each of the two periods (on days 5 and 10 of the experiment). Oxygen consumption, ammonia excretion and protein utilization were calculated by averaging measurements taken at the end of periods 1 and 2, because there were no significant differences between days for any of these parameters. Food consumption was measured on the last day of the experiment (see below) while growth and condition factor were calculated from the start of the experiment to the end of period 2.

To confine individual fish, a 'dummy' feeding apparatus (water-tight and air-tight), identical in appearance to the darkened feeding container, was inserted into the tank. Fish would enter in search of food. The lid was then closed (Fig. 1b), trapping the fish inside and the 'dummy' feeding container was placed in a water bath at 12 °C so as to serve as a thermostatted respirometer. Each fish (4 fish simultaneously) from each hierarchy was enclosed individually inside 'dummy' feeding containers in this fashion. From here, water samples (see below) were taken at hourly intervals for 6 h without disturbing the fish. All fish in a hierarchy were sampled at the same time.

Oxygen consumption was measured over the first hour during which the fish was held in the respirometer; the water was not aerated during this period. Water samples were analyzed using an oxygen electrode (Cameron Instrument, Port Aransas, Texas) thermostatted to the experimental temperature (12 °C) and connected to a Model 1900 A-M Systems Polarographic Amplifier digital dissolved oxygen meter (Carlsborg, Washington). After the first hour of holding, the water in the respirometer was gently aerated, and aeration continued for the next 5 h so as to maintain air saturation. This longer time period was required to obtain an accurate ammonia flux measurement (assay procedure modified from Verdouw et al. (1978)). At the end of 6 h, fish were individually anaesthetized in neutralized MS-222 (0.08 g L^{-1}), weighed, measured for fork length, and allowed to recover in their respective tanks.

The methodology of McCarthy et al. (1992) was used to determine individual food consumption. On the morning of day 11, fish were fed a ration of 1 % total tank weight of repelleted Martin commercial pellet feed (see Alves et al. (2006) for detailed description) containing 6 % (by mass of dry powdered food) Ballotini lead glass beads (0.400–0.455-mm; 8.5-grade, Jencons USA, Inc., Bridgeville, Pennsylvania). One hour after feeding, fish were terminally sampled with a concentrated dose of neutralized MS-222 (5 g L^{-1}) to cause quick euthanization without struggling. Fish carcasses were frozen at −20 °C until X-rayed (Faxitron 805 portable X-ray machine, Wheeling, Illinois; 1 s exposure at 70 kVP) to determine the number of glass beads ingested.

In a parallel study using identical methodology, 6 more hierarchies were established, with each fish having a clearly identified rank. These served as controls for toxicological experiments on hierarchy structure (Grobler and Wood, in preparation) and to provide plasma cortisol data for the present study. On day 11, fish from these additional hierarchies were quickly euthanized at 1 h after the final feeding using a concentrated dose of neutralized MS-222 (5 g L^{-1}). Blood was collected via tail severance, centrifuged to obtain plasma, and the decanted plasma was frozen immediately. Plasma cortisol concentrations were assayed without extraction with a Cayman Chemical EIA Kit (Ann Arbor, Michigan), according to the manufacturer's instructions, with appropriate dilutions to match concentrations in standards. The kit was validated against RIA, and via serial dilutions of trout plasma.

Calculations

During each morning feeding (8 in total—days 1–4 and 6–9), all fish were scored for aggressive behavior. Aggressive acts were not scored on days 5 and 10 as physiological measurements were conducted. Total aggressive acts for each fish were then divided by the 8 days of observations, to yield aggressive acts per day (each day representing a 15-min observation period).

To determine individual food consumption for a single meal, a conversion from glass beads to food consumption was accomplished by averaging the number of beads per pellet (see McCarthy et al. (1992), for detailed description), and counting the beads in each fish as a percentage of the total number of beads recovered.

Specific growth rate (SGR) was calculated as:

$$(\ln(BM_2) - \ln(BM_1))/(t_2 - t_1) \times 100, \tag{1}$$

where BM_1 and BM_2 were body masses at times t_1 and t_2, respectively.

Fulton's condition factor was calculated as:

$$\left(BM_{(g)}/L_{(cm)}^3\right) \times 100, \tag{2}$$

where BM is the weight and L is the fork length of the fish. Percent change in condition factor was calculated from the beginning of the experiment to the end. A positive percent change indicates increasing condition factor from the beginning to the end of the experiment.

Oxygen consumption rate (MO_2) was calculated as follows:

$$MO_2 = (\Delta PO_2 \times \alpha O_2 \times v)/(m \times t), \tag{3}$$

where ΔPO_2 (mmHg) is the measured change in PO_2 values between beginning and end of the first hour of physiological testing, αO_2 (µmol L^{-1} mmHg^{-1}) is the solubility constant for O_2 in water (Boutilier et al. 1984), v is the volume (L) of the 'dummy' feeding container, m (g) is the mass of the fish, and t is the time (h). A similar equation was used to calculate total ammonia-N excretion rate (M_{Ntotal}), substituting total ammonia−N for $\Delta PO_2 \times \alpha O_2$.

To calculate protein utilization, instantaneous relative use of protein as an aerobic metabolic fuel, the nitrogen quotient (NQ) was first calculated as outlined by Lauff and Wood (1996).

$$NQ = M_{Ntotal}/MO_2. \tag{4}$$

Protein use (PU), as a % of total aerobic metabolism, was then determined as:

$$PU = NQ/0.27, \tag{5}$$

where 0.27 is the theoretical maximum for NQ in a teleost fish where 100 % of aerobic metabolism is fueled by proteins (and amino acids) as derived by van den Thillart (1986).

Statistical analyses

Statistical analyses were conducted using SigmaStat 3.5 (Systat Software Inc. 2006), Statistica 7.0 (StatSoft Inc. 2004) and Statistical Package for the Social Sciences Statistics 19.0 (IBM 2011) software. Data have been analyzed in two different ways: (1) where physiological parameters were averaged on the basis of the fish's social status (i.e. rank in the hierarchy) and (2) where physiological parameters were plotted against the measured aggressive behavior of the individual. Probability levels have been reported throughout, and $p \leq 0.05$ was considered significant.

Social status and physiological parameters

As a first step, the mean data for each social rank in the hierarchies ($N = 7$) were compared between Periods 1 and 2, using Student's paired t test or the Wilcoxon's signed rank test in the case of data that could not be normalized by standard transformations. In the absence of significant differences, the data were averaged between Periods 1 and 2 before further analysis. One way repeated measures ANOVA tests followed by the Holm–Sidak post hoc test were performed to test for differences in oxygen consumption, ammonia excretion, protein utilization and feeding among the four ranks of social status. The repeated measures ANOVA was used because each of the separate hierarchies represents an independent replicate ($N = 7$), and the measurement (e.g. oxygen consumption) was repeated on each of the 4 fish in the hierarchy (i.e. on ranks 1, 2, 3, and 4). In this case, oxygen consumption was the "within subjects" factor and social rank was the "between subjects factor", and we report the significance of differences in oxygen consumption among the four ranks. Oxygen consumption data were log transformed and feeding data were natural log transformed to pass normality tests. Data on aggressive acts per day, growth, condition factor, and plasma cortisol could not be normalized by standard transformations, so differences for these parameters among the four ranks of social status were assessed by the Kruskal–Wallis test, a non-parametric version of ANOVA, followed by the Holm–Sidak post hoc test. Conclusions were unchanged when checked by the Friedman test, analogous to a non-parametric repeated measures ANOVA. In one case noted in Results, data from several social ranks were grouped together so that they could be evaluated by the Mann–Whitney U test. Possible differences in variability among social statuses were assessed

using Levene's test. Each social status rank contained seven individuals, one from each experimental hierarchy ($N = 7$, except for cortisol, where $N = 6$), and data were expressed as mean ± 1 SEM.

Individual behavior and associated physiological parameters of individuals

Measures of individual behavior and resulting physiology for each fish in each of the 7 experimental hierarchies (total $N = 28$ individuals) were analyzed by the Spearman's rank correlation. Scatter plots were created of individual feeding, growth, percent change in condition factor, oxygen consumption, ammonia excretion, and protein utilization against aggressive acts per day, and lines were fitted by least squares regression. In all cases, significance was assigned based on the rejecting the null hypothesis at $p = 0.05$.

Results

Behavioral measurements

Fish quickly entered the feeding container when it was inserted. The dominant fish spent the most time in the feeding container and consumed the majority of the food, while the other fish of lower social rank would enter sporadically to retrieve what was left over. At times, two fish cohabitated the feeding container simultaneously, with the subordinate fish leaving without any aggressive display from the dominant fish. In two additional trials, which were not used in the data presented, fish behaved similarly when the clear feeding container was inserted, and observations indicated that no aggressive acts took place inside the feeding container itself.

Dominance hierarchies were formed in all experimental groups, with six of the seven establishing stable hierarchies (consistent ranking over two successive days) in period 1. One tank needed 8 days of observation before a consistent rank could be determined. It was in this experimental group where the only observed rank 'switch' occurred: rank 1 and rank 2 fish swapped social status after period 1, becoming rank 2 and rank 1, respectively, in period 2. There was no relationship between starting weight or length or condition factor and final social status.

Overall, rank 1 fish exhibited significantly more aggressive acts than ranks 2, 3 or 4 (Fig. 2). Ranks 2 and 3 also differed significantly. In decreasing order, rank 1 exhibited 62.5 %, rank 2 showed 28.3 %, rank 3 displayed 7.3 % and rank 4 accounted for 1.9 % of aggressive acts per day.

Aggressive acts per day for each hierarchy did not differ between periods 1 and 2 (21.9 and 18.2, respectively). Three hierarchies displayed more aggressive acts in period
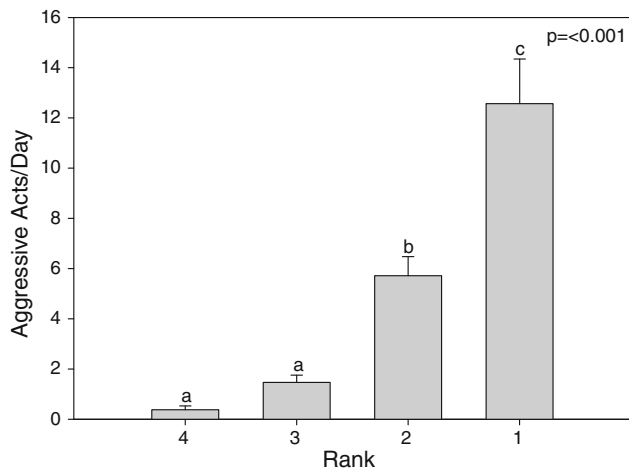
Fig. 2 Aggressive acts per day based on the social status. Values are mean ± SEM: $N = 7$ experimental groups. *Different letters* denote significant differences among ranks ($H = 23.9$; degrees of freedom = 3; $p \leq 0.001$; Kruskal–Wallis one-way ANOVA; post hoc test Holm–Sidak)

1 than in 2 (25.7, 40.5, and 31.2 compared to 16.0, 18.2 and 9.0 aggressive acts per day). However, in the other four hierarchies, the opposite was observed (14.0, 9.8, 14.7, and 17.5 during period 1 as compared to 30.0, 15.5, 16.5, and 22.2 aggressive acts per day in period 2). There was also no significant difference among different hierarchies in overall mean aggressive acts per day, the values ranging from 12.6 to 29.3 per day (overall mean across all hierarchies = 20.0).

Physiological measurements

Feeding

Feeding was quantified for a single meal on day 11. Dominant rainbow trout (rank 1) consumed the most food, eating 66.9 % of the total meal (Fig. 3a). The other trout of lower social status (ranks 2, 3 and 4) consumed significantly lower amounts, 23.2, 5.7 and 4.2 %, respectively. There were no significant differences among ranks 2, 3 and 4.

There was a highly significant positive correlation between feeding percentage and rates of aggression (Fig. 3b). Some fish with low aggressive acts did not consume any food, while fish with high aggressive acts consumed more food, regardless of rank.

Growth

Rank 1 fish had significantly higher specific growth rates than rank 2, 3, and 4 fish, with no difference being observed among the three lower social status fish (Fig. 4a).

Individual fish with higher aggressive acts had higher specific growth rates compared with fish displaying lower aggressive acts, regardless of social status. A positive and
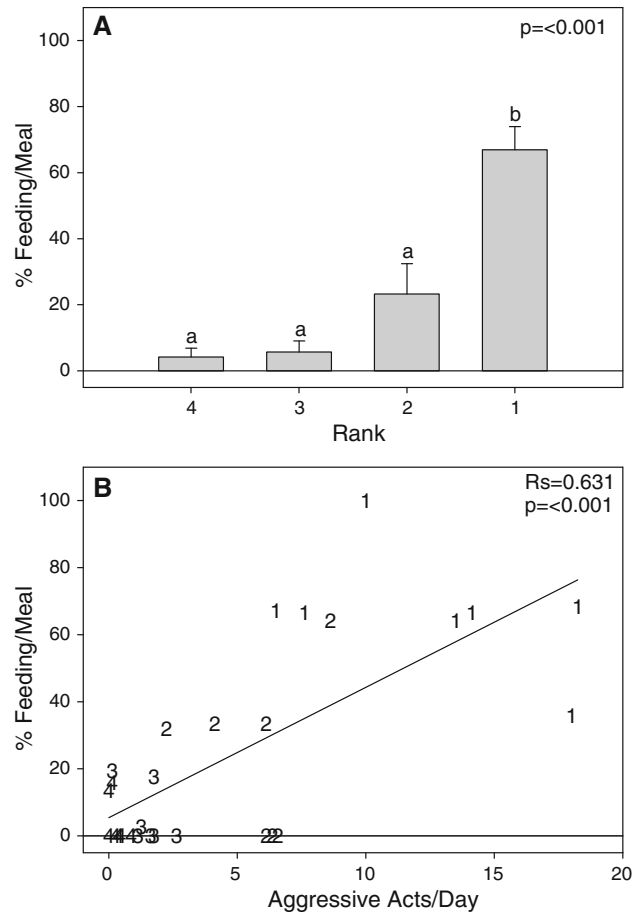
Fig. 3 a Percent feeding for one meal based on the social status. Values are mean ± SEM: $N = 7$ experimental groups. *Different letters* denote significant differences among ranks ($F = 16.7$; degrees of freedom = 3; $p \leq 0.001$; Repeated measures one-way ANOVA; post hoc test Holm–Sidak). **b** Relationship between percent feeding of one meal and aggression per day. Percent feeding for one meal and averaged total aggressive acts ($N = 28$; Spearman's rank correlation; $Rs = 0.631$; $p \leq 0.001$)

highly significant correlation existed between specific growth rate and aggressive acts in individual trout (Fig. 4b).

Condition factor

Percent change in condition factor was significantly higher in rank 1 compared to other ranks. Ranks 2 and 3, and 4 had similar percent changes in condition factor (Fig. 5a). There were no significant differences among the lower ranked fish (ranks 2, 3 and 4).

A strong positive correlation was observed between percent change in condition factor and aggressive acts per day (Fig. 5b). Fish tending to have high aggressive acts per day also exhibited high positive percent changes in condition factor as compared to fish showing low aggressive acts per day.
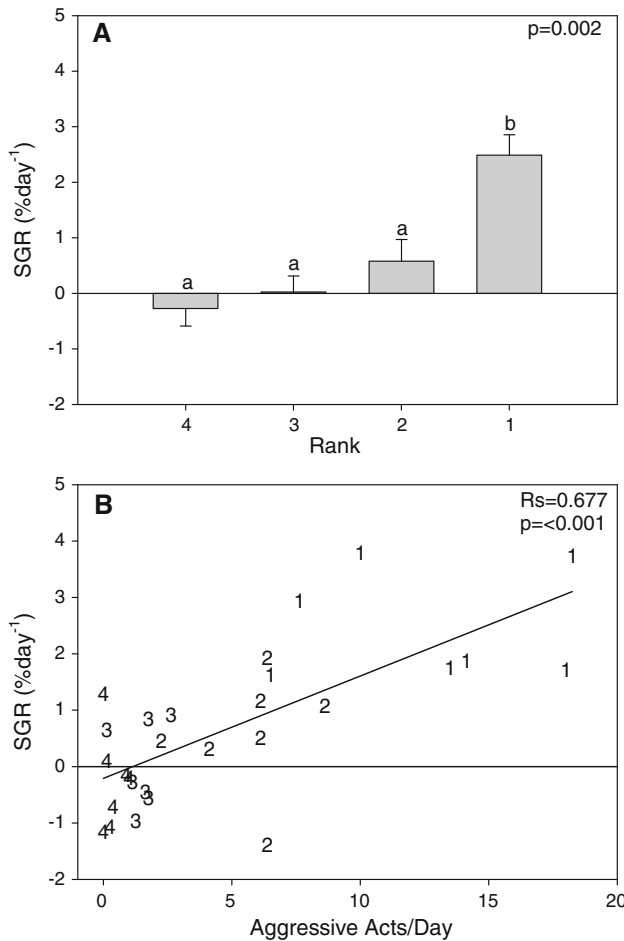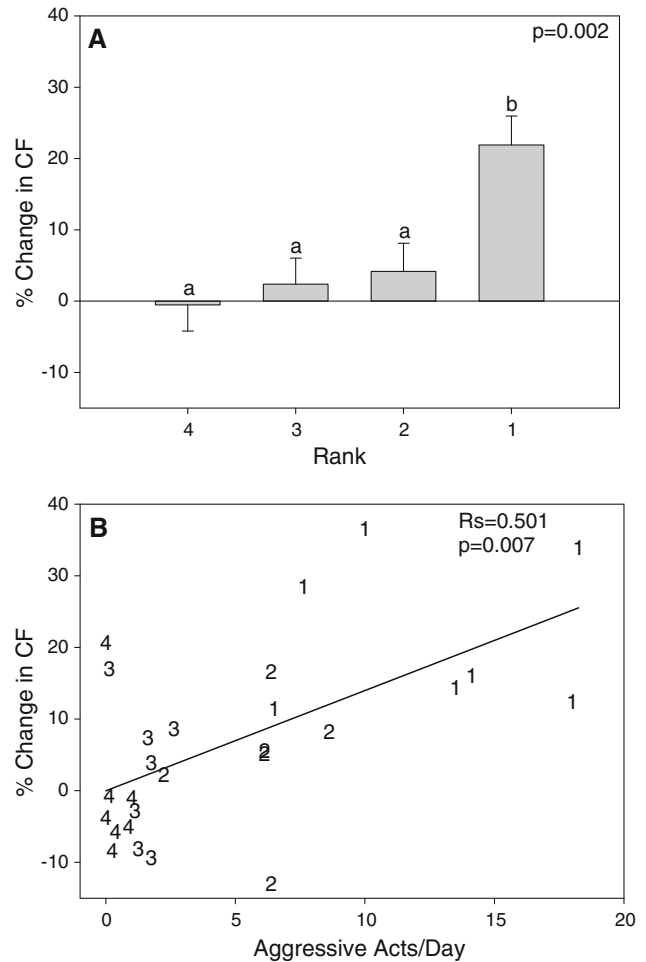
Fig. 4 a Specific growth rate per day based on the social status. Values are mean ± SEM: $N = 7$ experimental groups. *Different letters* denote significant differences in specific growth rates per day among ranks ($H = 16.8$; degrees of freedom $= 3$; $p = 0.002$; Kruskal–Wallis one-way ANOVA; post hoc test Holm–Sidak). **b** Relationship between specific growth rate and aggression per day. Specific growth rate and averaged total aggressive acts. $N = 28$ (Spearman's rank correlation, $Rs = 0.677$, $p \leq 0.001$)

Fig. 5 a Percent change in condition factor based on the social status. *Different letters* signify statistical differences among ranks. Values are means of ± SEM: $N = 7$ experimental groups ($H = 11.525$; degree of freedom $= 3$; $p = 0.002$; Kruskal–Wallis one-way ANOVA; post hoc test Holm–Sidak). **b** Correlation between percent change in condition factor and aggression per day. Percent change in condition factor from start of the experiment to the end of period 2 ($N = 27$; Spearman's rank correlation; $Rs = 0.501$; $p = 0.007$)

Oxygen consumption rate

Rank 1 had the lowest routine oxygen consumption, 13.4 μmol-O_2 g^{-1} h^{-1}, while ranks 2, 3 and 4 had similar oxygen consumptions which were about 70 % higher: 22.6, 23.9, and 23.2 μmol-O_2 g^{-1} h^{-1}, respectively (Fig. 6a). There were no significant differences among the four ranks by repeated measures ANOVA. However, when all ranks of lower social status (rank 2, 3 and 4) were combined and compared to rank 1 fish, rank 1 had a significantly lower rate of oxygen consumption than the combined subordinates (Mann–Whitney U statistic, $p = 0.017$).

There was also no significant correlation between oxygen consumption and aggressive acts per day in individual fish (Fig. 6b). Note that fish of lower rank exhibited more variable MO_2 values than dominant fish, however, this

difference in variability was not statistically significant (Levene's test, $p = 0.109$).

Ammonia excretion rate

Ammonia excretion was the highest in rank 1 fish, about 70 % greater than in ranks 2, 3 and 4 (Fig. 7a). There were no significant differences among ranks 2, 3 and 4.

A significant positive correlation was observed between ammonia excretion and aggressive acts per day in individual fish (Fig. 7b), with fish displaying high aggressive acts tending to have higher ammonia excretion rates compared to fish that exhibited low aggressive acts.
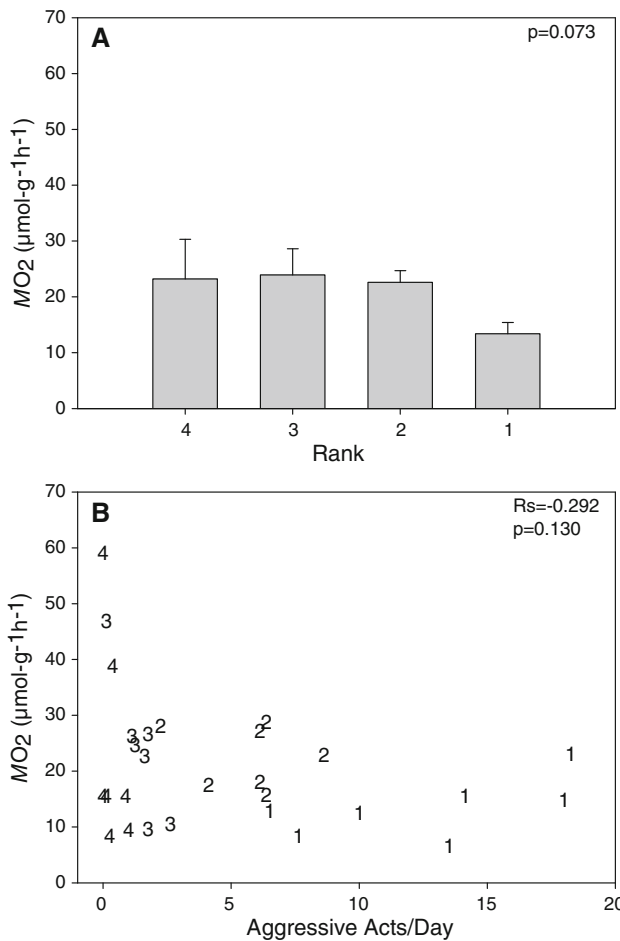
Fig. 6 **a** Oxygen consumption rates based on the social status. Values are mean ± SEM: $N = 7$ experimental groups ($F = 2.74$; degrees of freedom = 3; $p = 0.073$; Repeated measures one-way ANOVA; post hoc test Holm–Sidak). **b** Oxygen consumption and aggression per day. Oxygen consumption and averaged total aggressive acts ($N = 28$; Spearman's rank correlation; $Rs = -0.292$; $p = 0.130$)

Protein utilization

Rank 1 had the highest percent protein utilization in aerobic metabolism (48.7 %), a value that was significantly higher by 2 to 3-fold than in all the other ranks. Percent protein utilization did not differ significantly among ranks 2, 3 and 4: 15.5, 26.4 and 17.5 %, respectively (Fig. 8a).

Percent protein utilization exhibited a significant positive correlation with aggressive acts per day in individual trout (Fig. 8b). Fish displaying lower aggressive acts tended to have lower percent protein utilization when compared with those displaying higher aggressive acts.

Plasma cortisol

Plasma cortisol concentrations were measured in fish on day 11 from a parallel set of hierarchies established under identical conditions. There were no significant difference
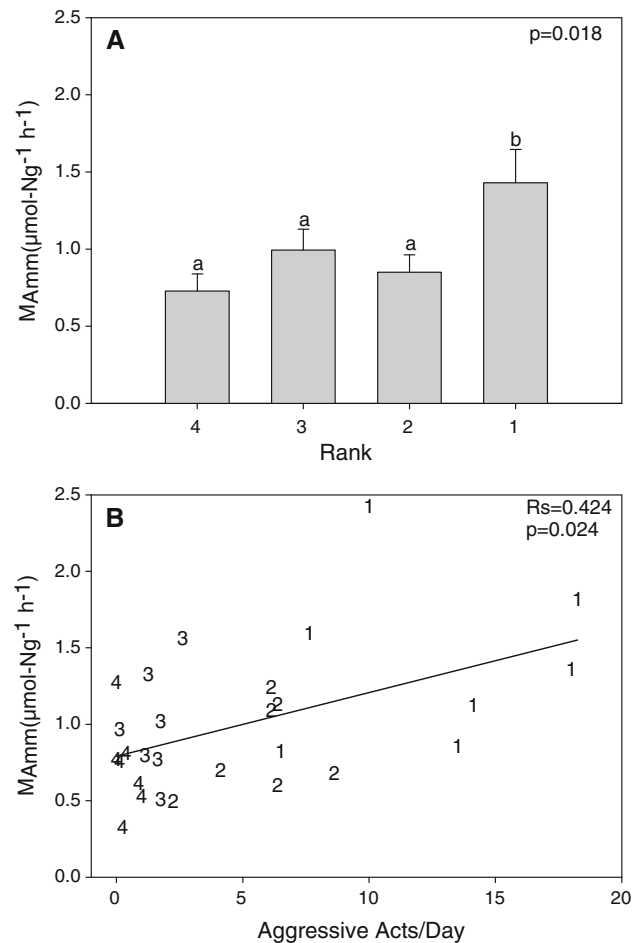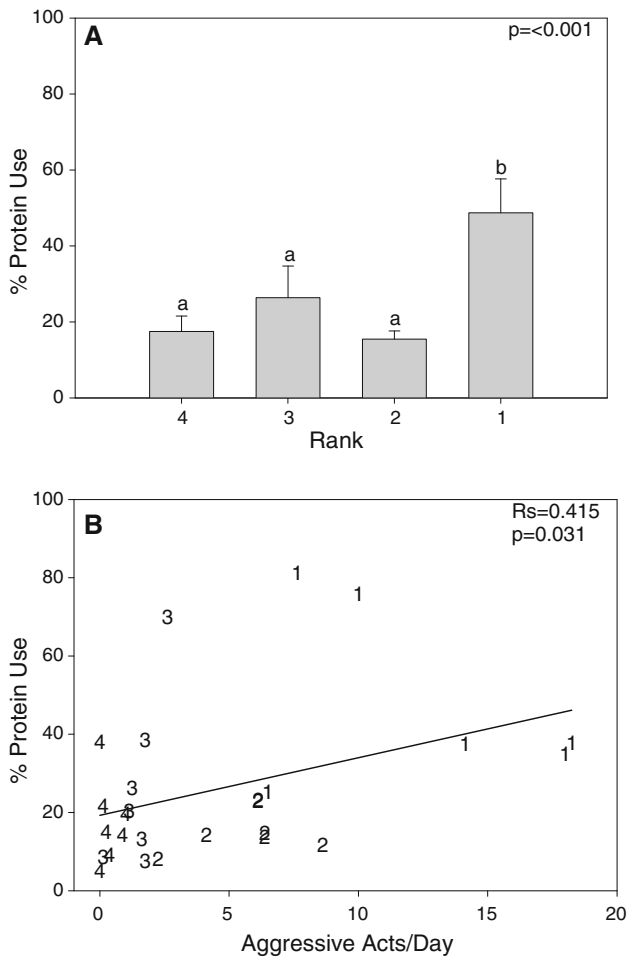
Fig. 7 **a** Ammonia excretion rates based on the social status. *Different letters* signify statistical differences among ranks. Values are mean ± SEM: $N = 7$ experimental groups ($F = 4.35$; degree of freedom = 3; $p = 0.018$; Repeated measures one-way ANOVA; post hoc test Holm–Sidak). **b** Correlation between ammonia excretion and aggression per day. Ammonia excretion and averaged total aggressive acts ($N = 28$; Spearman's rank correlation; $Rs = 0.424$; $p = 0.024$)

between ranks 1, 2, 3, and 4, but variability was statistically greater in the lower social status ranks as compared to dominant fish (Levene's test, $p = 0.025$, Table 1).

Discussion

Behaviour

Aggression was the only behavioral measurement needed to assign social status to individual fish. All groups of fish formed linear dominance hierarchies within 8 days, which is consistent with most previous dominance studies in fish (see Sloman and Armstrong 2002, for a comprehensive review). The level of aggression per hierarchy did not differ between periods 1 and period 2 ("Behavioral measurements"). This is not a surprising finding. Brown trout

Fig. 8 a Percent protein use based on the social status. *Different letters* signify statistical differences among ranks. Values are means of $\pm$ SEM: $N = 7$ experimental groups ($F = 9.00$; degrees of freedom $= 3$; $p \leq 0.001$; Repeated measures one-way ANOVA; post hoc test Holm–Sidak). **b** Correlation between protein use and aggression per day. Percent protein use and averaged total aggressive acts ($N = 27$; Spearman's rank correlation; $Rs = 0.415$; $p = 0.031$)

Table 1 Plasma cortisol concentrations (means $\pm$ 1 SEM) of fish held in hierarchies of four individuals based on the social status ($n = 6$)

Social Status	Plasma cortisol (ng/ml)
1	59.5 ± 8.8
2	88.2 ± 33.8
3	130.0 ± 46.1
4	116.1 ± 57.9

Blood plasma for plasma cortisol measurements were collected after final feeding (day 11). There were no significant differences ($H = 1.89$; $p = 0.59$; Kruskal–Wallis one-way ANOVA)

in hierarchical groups of four also exhibited consistent daily aggression over 2 weeks (Sloman et al. 2000a). However, aggression decreased across days in salmon and rainbow trout (Brown and Brown 1993) and, in zebrafish, aggression increased across days (Filby et al. 2010). Possible explanations for these different time-dependent patterns could include differences in the size of the tanks and/ or in the time schedule of feeding and behavioral measurements. In the present study, the relatively high loading density (4 fish/30 L tank) may have helped maintain aggression levels over time. In addition, as in Sloman et al. (2000a), behavioral measurements were conducted during and immediately after feeding, a time when fish not only have to compete to gain access to food, but also must consume the food. In contrast Brown and Brown (1993) and Filby et al. (2010), videotaped behavior before feeding occurred when fish were fighting for position, and thus could allocate as much time and energy to aggression as needed. This suggests that there might be a limit for how much aggression can occur during a feeding event, yielding consistency over time. However, our initial observation that aggression levels were similar in two hierarchies between morning (feeding container present) and afternoon sessions (feeding container absent) argues against this interpretation. Few studies report aggression levels throughout their experiment and overall, definitive conclusions are difficult to draw on this issue.

Physiology

Our first hypothesis that each social rank would display a distinct physiological profile, different from the other ranks, was not supported. When the physiological data from the 7 different hierarchies were grouped according to rank, there appeared to be only two 'classes' of physiologically different fish amongst the four fish in each hierarchy: dominant individuals that consumed a higher percentage of food, had higher growth rate, higher percent change in condition factor, higher ammonia excretion, higher protein utilization and lower routine metabolic rate (i.e. O_2 consumption) as compared to the three other fish that became subordinate individuals and had similar physiology to each other. This suggests that the dominant fish is favored in such a competitive setting, with the three subordinate fish having equal, less favorable physiologies. This led us to explore another way of analyzing the same data, taking into account individual behavior rather than "binning" the data according to social rank. When the physiological data were plotted as a function of individual behavior, linear correlations occurred between aggressive acts and most measures of the resulting physiology, suggesting a continuum of physiological responses related to aggression levels. Although not the first (c.f. Sloman et al. 2008), to the authors' knowledge, the present study is one of the few to compare conclusions based on data grouped according to social rank with conclusions based on data

related to individual aggression scores in social hierarchies containing multiple fish. The implications of the two different methods of data analysis are explored in the following "Implications of two different methods of data analysis".

Our second hypothesis, that dominant fish would rely less on protein and amino acid metabolism, so as to direct more nitrogen into protein growth, was also not supported. Instead, the rank 1 fish exhibited the lowest metabolic rate, the highest ammonia excretion rate, and therefore the greatest protein utilization. Ranks 2, 3, and 4 displayed lower ammonia excretion rates, higher oxygen consumption rates, and therefore lower protein utilization (Figs. 7a, 8a). Fish fed to satiation are known to have higher ammonia excretion rates compared to unfed fish (Alsop and Wood 1997; Bucking and Wood 2008), but those measurements were taken within 12 h of feeding. In the present study, physiological parameters were taken at 24 h+ post-feeding, when ammonia excretion rates should have returned to post-absorptive values (Brett and Zala 1975; Secor 2009). Therefore, the surprising conclusion is that dominant individuals were burning relatively more muscle protein for energy, even though they were at rest during the actual measurements. A possible additional cause could be increased ATP turnover and associated degradation immediately prior to the period of respirometry, because Mommsen and Hochachka (1988) showed that working muscle in rainbow trout produces ammonia through deamination of adenylates. This suggests that dominant individuals may be somewhat inefficient in fuel use, using muscle protein and adenylates as a fuel source—i.e. they are able to build more muscle, but "waste" some of it in maintaining dominance. This is likely a necessary trade-off, between protein growth and maintaining a high social status by continuing aggressive behavior.

Dominant fish used aggression to monopolize food, restricting the consumption of other fish (Fig. 3a), in agreement with previous studies (Sloman et al. 2001; Nicieza and Metcalfe 1999). These results suggest that monopolizing food has a positive effect on growth rate (Fig. 4a). However, it is not known if dominant fish consumed the majority of food every day, since food consumption was only measured once, at the end of the 10-day experiments. Dominant fish also exhibited lower routine rates of oxygen consumption (Fig. 6a) and a tendency for lower cortisol levels (Table 1). Cortisol is known to reduce growth rates, increase metabolic expenditure, and reduce competitive ability in salmonids (Gregory and Wood 1999a, b; De Boeck et al. 2001). The complete data set suggests that high feeding rate, low metabolic rate, and low cortisol all contributed to the higher growth rates observed in dominant individuals.

The higher percent change in condition factor in dominant fish may also be attributed to differences in feeding (Fig. 5a). Condition factor can be used as an index for fish health (Gilmour et al. 2005). Fish with high food consumption can have greater energy reserves and repair damage more easily. Gregory and Wood (1999a) reported that trout which consumed a higher ration in a hierarchy had a higher fin condition index compared to individuals who ate less. Sloman et al. (2000b), examining hierarchies of four brown trout, reported a different result: rank 2 individuals had a negative change in the condition factor, while ranks 1, 3 and 4 had positive, non-significant changes in condition factor. Possibly, this difference is attributable to the relatively complex semi-natural stream environment used by Sloman et al. (2000b) as compared to the more uniform laboratory setting of the present investigation.

Lower routine metabolic rate (RMR) was observed in dominant fish (see "Oxygen consumption rate"). Notably, the current measurements of routine MO_2 were taken 24+ h post-feeding when specific dynamic action effects should be minimal (Brett and Zala 1975; Secor 2009), in a situation where the fish are not interacting with one another, but rather sitting quietly in a darkened respirometer. This does not fit with evidence that Atlantic salmon with high resting metabolism tend to achieve higher social status (Metcalfe et al. 1995; McCarthy 2001). However, our measurements of routine MO_2 were taken after hierarchy formation, and as such, our findings fall inline with the past research done by Sloman et al. (2000c) where dominant brown trout had lower standard metabolic rate (SMR) than subordinates after being confined in pairs. Thus, once established, dominance is a predictor of lower RMR and SMR. Dominant individuals expend less energy while consuming the highest percentage of food.

Van Leeuwen et al. (2011) recently reported that higher food consumption caused an elevation of SMR in juvenile coho salmon. However, the coho salmon were not held in dominance hierarchies, where stress-induced cortisol elevation could be a factor. This is potentially another reason for the difference seen in the current study and that of Sloman et al. (2000c), in comparison to the results of Van Leeuwen et al. (2011). Elevated plasma cortisol may be both a cause and a consequence of lower social status in salmonids (Gregory and Wood 1999b; Gilmour et al. 2005). Individuals of lower social status in laboratory settings may have elevated plasma cortisol levels (Abbot and Dill 1989; Fox et al. 1997) which could cause increased oxygen consumption in trout (Sloman et al. 2000c; Morgan and Iwama 1996; De Boeck et al. 2001). In our studies on trout from a parallel separate set of hierarchies, cortisol levels tended to be higher and significantly more variable in fish of lower social rank, but none of the differences in the means were significant (Table 1).

Implications of two different methods of data analysis

Two physiologically distinct groups were observed when the data were grouped according to rank of social status in dominance hierarchies. However, investigating the individual behavior produced a positive, linear correlation between most physiological parameters and aggressive acts per day. This suggests that there are two possible methods for interpreting a social hierarchy, which can lead to different conclusions.

Although not incorrect, grouping individuals from different hierarchies together based on their respective social status can produce inherent problems. Each hierarchy consists of a different set of fish and thus each hierarchy will be different. Considering this, Briffa and Elwood (2010) suggest using repeated measures statistics to analyze the group data, and indeed this was the method used in the present study. This approach is valid from a statistical point of view as it measures differences between ranks taking into consideration different experimental groups (i.e. different hierarchies). However, the approach tends to obscure the variability of both physiological and behavioral measurements. This is due to individual behavior affecting physiology. Since each hierarchy consists of different fish, each hierarchy will inevitably have a different behavioral environment. Each fish will then adjust its behavior based on its unique environment. For example, in a high aggression hierarchy, rank 4 fish might perform two aggressive acts per observation period, while a rank 4 fish in a less competitive hierarchy might display zero aggressive acts or vice versa. The two fish are in different environments and are thus behaving differently with different resulting physiology. However, when social status is assigned, these fish are grouped together. This creates a potential "statistical artifact" and information content on individual behavior is masked.

However, when physiological data are plotted against aggressive acts per day, a linear correlation is seen between many physiological measures and behavior regardless of assigned social status (Figs. 3b, 4b, 5b, 7b, 8b). As aggression per individual varies so does the resulting physiology, and the pattern represents a continuum. This conclusion is 'hidden' when the data are grouped according to social rank. An interesting exercise is to re-analyze these relationships based on the three subordinates classes only, where "binning" revealed no differences. All the relationships became weaker, which is not surprising based on the reduced N and the greater physiological similarity of the subordinates to each other. Nevertheless, the signs of correlations remained unchanged, and two of them (SGR and % feeding/meal) remained statistically significant, reinforcing our conclusion as to the additional information value of this approach.
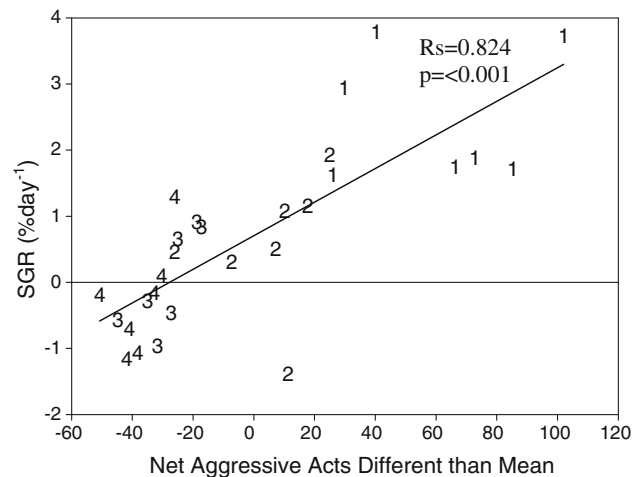
Fig. 9 Relationship between specific growth rate and net aggressive acts different from hierarchy aggressive mean. Mean aggressive acts for each hierarchy was calculated. Next, aggressive acts performed by each fish was subtracted from their respective hierarchical mean. *Positive values* indicating fish displayed more aggressive acts than the hierarchical mean and *negative values* representing fish that displayed less aggression than their hierarchical mean. ($N = 28$, Spearman's rank correlation, $Rs = 0.824$, $p \leq 0.001$)

To further illustrate that aggression per fish reveals a more complete story, aggression levels per hierarchy were standardized by calculating the mean aggressive acts per hierarchy and measuring the difference of each fish from that mean (i.e. a positive value indicating that fish performed more aggressive acts than the mean in their respective hierarchy, and vice versa). As seen in the example of Fig. 9, in comparison with Fig. 4, a stronger correlation exists between specific growth rate and individual aggressive acts and specific growth rate when the level of aggression in each hierarchy is controlled ($Rs = 0.824$ vs. $Rs = 0.677$). Similar differences (i.e. stronger linear correlations) were seen when the same analytical approach was applied to feeding, condition factor, oxygen consumption, ammonia excretion and protein utilization (data not shown). This shows that physiology of fish in a dominance hierarchy is affected by the individual's specific behavior in response to its unique behavioral environment.

Some previous studies have related individual behavior and individual physiology, but these mainly examined only pairs of fish over a short period (see "Introduction"). However, in a similar study, Sloman et al. (2008) successfully correlated individual specific growth rate of brown trout to individual aggression in social hierarchies consisting of 12 fish in a semi-natural environment. More aggressive displayed higher specific growth rate compared to fish that were not as aggressive. However, the relationships were not seen with physiological means based on the social status. Once individual behavior was taken into consideration, clear trends emerged, similar to the current study.

In conclusion, the importance of linking behavior and physiology on a continuous scale is not just a statistical issue. We suggest that this approach be more widely applied to social hierarchies consisting of more than two individuals. As this study illustrates, there is a wealth of information that is potentially masked during the 'binning' process. The richness of the interplay between aggressive behavior and physiology may be obscured by just calculating means and comparing across status ranks, and valuable information on mechanisms of linkage may be lost. In addition, if individual variation is distributed along a continuous scale, it informs on topics such as "personality" and "coping styles". And, last but not least, it must be remembered that natural selection acts on variance in the phenotype, not on mean characteristics.

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