UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

# SEX-SPECIFIC BEHAVIOR IN JUVENILE BROWN TROUT (SALMO TRUTTA)

Investigating the impact of sex on dominance and aggression among juvenile brown trout by using new DNA-based sex identification.

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# Abstract

In salmonid populations, social structures generate dominant individuals that typically display higher aggression, securing high-quality resources and optimize chances of survival. Studies within the subject of dominance and sex-specific behavior within adult salmonid groups concludes that salmonid males usually take on the dominant role, likely due to distinct reproductive roles between the sexes. Similar previous research on juvenile salmonids is limited and instead indicates that males are also more aggressive, but not consistently more dominant. This study addresses this gap in knowledge by combining a short-term dominance assessment using an artificial stream and 96 brown trout captured by electric fishing around Lunz am see, Austria, with a non-lethal DNA sex identification method using DNA from the same individuals. The study explores how residency during 10-day rounds and time of day influences the overall aggression level and when dominance is established. Additionally, it investigates how sex and group body size influences dominance and aggression. The result indicates that both residency and time of day were significantly affecting the overall aggression, with higher levels at dawn, consistent with earlier studies. The interaction of day and time also significantly affected the establishment of dominance, with early dominance also visible at dawn, and were overall stable from day 8. Both sexes became dominant within the groups, but the aggression in groups with female dominant individuals significantly increased with group body size, opposite to earlier studies. These findings may provide insight into how various factors can influence aggression and dominance in juvenile brown trout populations, which could help develop strategies for their conservation.

# Sammanfattning (Swedish abstract)

Laxpopulationer skapar sociala strukturer med dominanta individer som uppvisar högre aggressionsnivåer för att säkerställa tillgången till högkvalitativa resurser och därmed optimera sina överlevnadschanser. Tidigare studier om dominans och könsspecifika beteenden har påvisat att vuxna hanar är mer dominanta och aggressiva än honor, troligen på grund av deras produktiva roller. Liknande studier med juvenila laxarter är få och indikerar i stället att juvenila hanar också är mer aggressiva, men inte konsekvent mer dominanta. Syftet med denna studie är att fylla denna kunskapslucka genom att kombinera en korttidsbedömning av dominansroller och aggression uppdelat i 10-dagars experimentella rundor. Detta utfördes i ett konstgjort vattendrag med 96 juvenila öringar fångade med elfiske runt Lunz am See i Österrike. Experimentet kombinerades med en ny icke-dödlig molekylär metod för könsidentifiering med DNA från fenor. Studien utforskar hur antal dagar och tid på dagen påverkar aggressionsnivån och etableringen av dominanta roller i grupperna. Dessutom undersöktes hur kön och kroppsstorlek påverkar dominansroller och aggression. Resultat indikerar att en kombination av antal dagar och tid på dagen har en signifikant inverkan på aggression med ökade nivåer vid gryning, vilket överensstämmer med tidigare studier. Interaktionen mellan antal dagar och tid på dagen visade sig också ha en signifikant påverkan på etablering av dominans, med tydligare etablering vid gryning och en stabil etablering efter dag 8 i alla sektioner och rundor. Båda könen visade sig bli dominanta, men aggressionsnivåerna ökade med dominanta honor och ökad kroppsstorlek inom gruppen. Resultaten från denna studie ger insikt i hur olika faktorer påverkar aggression och dominans hos juvenila öringspopulationer, vilket kan hjälpa till att utveckla strategier för deras bevarande och återetablering.

# Introduction

Individuals regularly demonstrate variations in behavior, commonly referred to as personality traits (Réale et al. 2007). A variety of personality traits have been identified across a wide range of animal species and within the same species and group (Soto, 2018). Understanding why individuals of the same species exhibit various behaviors involves separating a complicated relationship of underlying factors, some correlated, which all influence behavior. These factors contribute significantly to evolutionary adaptations, including sexual selection (Parker & Pizzari, 2015). Several personality traits can often be linked together, such as aggressiveness with boldness (Huntingford, 1976) and a higher affinity to investigate the surroundings (Bell, 2005). Personality traits often determine social structures, assigning individuals to dominant or subordinate roles. Dominance often involves one animal displaying more aggression towards another (Drews, 1993) to secure better long-term access to vital resources like food, water, habitats, and mating opportunities (Kaufmann, 1983). These hierarchies not only shape interactions within groups but also control resource division to other group members through continuous hierarchy (Chase et al., 2002). Dominance can also occur in short-term contests where the winner instantly gains resources or access to mating, without setting a continuous hierarchy within the group (Drews, 1993). Once established, dominant individuals convey their status through visible cues, making it easier for others to recognize their rank and uphold their dominance by engaging in minor aggressions (O'Connor et al., 1999).

Salmonids, native to the northern part of the hemisphere (Crawford & Muir, 2008) stand out as one of the most extensively researched taxa within this field (Conrad et al., 2011; Mittelbach et al., 2014), and these studies have considerably furthered our understanding of how different variations in natural selection can influence personality traits (Conrad et al., 2011). This knowledge is fundamental when working with ecosystem conservation and restoration, since salmonids are vital to the ecosystems as key predators. They also support river ecosystems by depositing nutrients from the ocean (Helfield & Naiman, 2006). During recent years, the salmonid rate has declined significantly (Thorstad et al., 2021). This decline is believed to be the result of a combination of many different factors. Some of these factors are the substantial environmental pressure due to their exploitation in commercial and recreational fishing activities (Gresswell & Liss, 1995), the ongoing destruction of suitable habitats for the different life stages (Foote et al., 2020) and migration barriers caused by hydro power plants (Fjeldstad et al., 2018).

## Habitation and social strategies

To gain further understanding of their behavior and to improve the conservation of these important salmonid species, numerous studies have examined individual-level social hierarchies, focusing on different specific individuals separately. This has usually been done by assessing short-term dominance by observing interactions within small groups over brief time periods, typically lasting a few days (Tiira et al., 2009). This approach has proven effective, yielding valuable understandings of the social hierarchies of salmonids and has shown that in freshwater sites, salmonid habitats with higher complexity, which include elements such as dead wood and larger stones, are shown to positively impact the overall population density of both adults and juveniles (Floyd et al., 2009). This complexity contributes to increased food production, provides a shield against predators (Coulston & Maguhan, 1983) and reduced visualization between individuals in inter- and intraspecific competitions, leading to a decrease in aggression and competition for food (Sundbaum & Näslund, 1998). Salmonid species are generally very territorial and commonly establish and protect their respective territories through aggressive displays, such as attacking, biting and chasing intruders. This usually leads to one individual outcompeting rivals and becomes the most dominant with the micro-habitat with highest complexity to ensure further chances of survival and reproduction (Amarasekare, 2002; Case & Gilpin, 1974). The swimming capacity of salmonids is shown to be correlated with the size of the fish, meaning that juveniles have limited swimming abilities. As a result, they are often restricted to pools and calmer waters near their hatching sites (Ojanguren & Braña, 2003). This restriction leads to groups of juveniles occupying the same small areas, with an increase in altercation and competition for habitats to enhance their chances of surviving their first year. Dominance in these groups is often linked to elevated activity (Adriaenssens & Johnsson, 2013; Johnson & Näslund, 2018) and metabolic rate (Metcalfe et al., 1995), enabling individuals to attain and defend valuable territories (Cutts et al., 2002). The later increase in fork length allows them to venture further away and can therefore more easily avoid altercation with bigger individuals (Ojanguren & Braña, 2003). To establish and maintain social hierarchies, avoid injuries and decrease energy expenditures, salmonids can visually signal their ranking to other individuals by rapidly changing their body coloration. In one study, the body coloration of individuals losing territorial competitions became darker as a sign of submission, while the winners preserved their original lighter coloration. This change typically resulted in a decrease in aggressiveness from the dominant fish (O'Connor et al., 1999). Compared to individual level studies on salmonid, studies focusing on salmonid behavior at a population level, where assessment of the behavioral dynamics of the whole group have occurred, are instead limited. Previous studies have found a positive relationship between growth rate and aggression, highlighting the overall dynamics of the group (Kadri et al., 2005) and between time of residency and aggression (Lahti et al., 2001). Additionally, groups of salmonids also tend to exhibit heightened activity at dawn, which is believed to coincide with the re-establishment of social order after darkness (Kadri et al., 2005). Other previous studies indicated that aggression was instead higher in the daytime with a decrease overall activity level during nighttime, which can specify sleeping behavior, such as distinct resting posture, regulation of homeostatic mechanisms and elevated threshold for arousal in response to sleep deficiency (Fraser et al., 1993).

## Sex-specific personality traits in juvenile salmonids

One biological factor that fundamentally shape personality traits of most animals is sex (Trivers, 1972). Sex-specific differences, often tied to variations in sperm and egg size (so-called anisogamy) leads to females normally allocating more energy reserve to gamete production than males (Hayward & Gillooly, 2011; Trivers, 1972). Consequently, the fecundity of females is commonly limited by their production of gametes, while the fecundity of males is limited by their accessibility to females (Trivers, 1972). Such a scenario can utilize substantial selection pressure on the size of the female body in fish since body size in females is closely linked to egg production and higher reproductive ability, meaning that larger females usually has a better ability to produce eggs (Barneche et al., 2018). Sex-specific behaviors in adult individuals can also be influenced by the process of sexual selection (Janicke & Morrow, 2019). Studies focusing on sex-specific traits in adult salmonids are abundant and clearly states that the behavior in salmonids is also known to vary between sexes, especially when both coexist within the same species and area, leading to different behaviors within the group (Magurran, 1993) and that males are more dominant and aggressive after sexual maturation than females (Weir et al., 2004; Jacob et al., 2007). Studies of sex-specific personality traits in juvenile salmonid species are in present time few and indicates that the juvenile male rainbow trout (Oncorhynchus mykiss) also displays an increased aggressivity in interspecific interactions (Sadangi, 2013). Similarly, juvenile brown trout males were observed to attack twice as much as females in interspecific interactions. However, there were no significant difference in number of wins between males and females, suggesting that the most dominant individual may not consistently be male (Johnsson et al., 2001).

## Sex-identification

To study sex-specific personality traits in salmonids, the determination of the sex has consistently posed a challenge. While it is possible to visually identify the sex of adult individuals in the field based on morphological indications, the visible identification of juveniles is instead impossible, since sexual dimorphism only becomes apparent after sexual maturation (Quémére et al., 2014). This has led to many studies of juvenile salmonids usually includes lethal identification through dissection of male and female sex gonads (Johnsson et al., 2001). To conserve juvenile salmonids, there is a vital need for non-lethal identification of sex that is both rapid and accurate. New advancements in molecular methods have provided other ways to determine sex in numerous taxonomic groups, for instance in salmonids (Morinha et al., 2012; Vanpé et al., 2013). In many salmonid species, it has been shown that the sdY gene (sexually dimorphic on the Y-chromosome 9) is a conserved sequence that is male-specific and tightly correlated to the sex-determining (SEX) locus. This has concluded that most salmonid species share a sex-determining (XY/XX) system with SEX locus being common. The new finding of conserved sdY in salmonid has led to the development of PCR tests for the sexing of different salmonid species with the use of agarose-gel electrophoresis and DNA samples. This new technique of using DNA for identifying sexes in salmonid species mitigates easier and more accurate molecular techniques to advance the knowledge of sex ratios at juvenile stages, as well as better ethical handling of the fish. It has also improved the conservation of viable water streams by being able to release the same juveniles back after sex-identification (Yano et al., 2012.

To conserve these species in the future and gain a healthier stream ecosystem, it is important to advance even further understanding of the behavior of juveniles (Furusawa & Koizumi, 2024). According to earlier studies, research exploring the interaction between aggression, dominance and other variables on a population level is specifically essential to bridge existing gaps in scientific knowledge (Kadri et al., 2005). Combining the investigation of behavior of juveniles and the influence of sex offers insight into their ecology and life history, which is crucial for sustainability in captivity and the wild (Johnsson et al., 2001). This knowledge also can help develop strategies to analyze viability and resilience (Quéméré et al., 2014) and improve conservation efforts influenced by migration tendencies and life history trade-offs (Lavender et al., 2023).

## Aim of study

To address the gap in research regarding sex-specific dominance and aggression in juvenile brown trout at a population level, as well as the need for a non-lethal method for sex identification in salmonids, this study integrates short-term dominance assessment on a population level from an artificial water stream over 10-day periods with the new DNA sex identification of the male sdY gene. The aim of the study is to investigate the overall differences in aggression within groups of juvenile brown trout throughout the experimental rounds and during different time intervals. Furthermore, the aim is to identify when dominance can be visible within the groups and whether this varies throughout the day. Lastly the aim of the study is to investigate whether sex and group body size in juvenile brown trout prior to sexual maturation influences dominance and aggression levels by identify the sex of the dominant individual within each group.

The study hypothesizes that:

- Both attacks and the number of attacked individuals will be affected by the day of round and time of day, with overall higher aggression levels observed at dawn (5 am), and lowest at nighttime. Initially, all time intervals throughout the rounds will show low aggression due to acclimation during the initial stage of the social dynamic, followed by a rise during dominance competition, and a subsequent decline upon dominance establishment.

- Visible establishment of dominance is affected by both days of round, with an increased visibility at dawn (5 am) with the re-establishment of dominance after darkness and will become evident at all or most time intervals within the 10-day rounds.

- Both males and females will establish dominant roles within the groups.

- Groups with males as established dominants will show a higher level of attacks than groups with female dominants.

- Both attacks and number of attacked individuals will be positively correlated by mean fork length of whole group and with males as the dominant individuals of the groups.

The findings of this study may be able to offer insights into how different temporal and dynamic factors can influence aggression and the establishment of dominance within juvenile brown trout population, which may help improv the conservation and re-establishment of these important salmonid species in different areas.

# Method

The practical parts were performed at Wasser Cluster, Lunz am See, Austria between 31/7-2023 and 7/9-2023 and was divided into two phases. Phase 1 was the short-assessment experimental behavior part at the artificial water stream “Hitec Flume” that was a part of a large Formas-funded project. Video analysis was used to analyze the dominant and aggressive behaviors of the test organisms within the different sections. Phase number 2 was the sex identification with PCR test of fin clips of the same test individuals after the behavior assessment at the Hitec flume. Lastly the result of the two phases was statistically analyzed using multi-linear regression models and type II Anova test.

# Ethical declaration

The experiments in this study were conducted in accordance with the terms of ethical approval. Ethical license number: GZ. 2023-0.053.856.

# Test organism

The test organisms used in this study were 96 brown trout (100-150 mm fork length) of unknown sex, captured using electric fishing from water streams in Kleiner Kamp, Lohnbach, Mendlingbach and Ois in Austria. After collection, the fish were anaesthetized using 2-phenox ethanol dissolved in water (0.5m/l). ID-tags (so called PIT-tags) were implanted that identified the movement of the fish with the use of scanners and antennas for registration of distinct identification number of the PIT-tags. Under anesthesia, fork length and weight were also measured along with images and the ID-number, before divided into groups of six individuals with similar fork length and placed in acclimatization pool for 24 h. After acclimatization, the fish were placed into the sections of the artificial stream Hitec Flume in the same groups in one 10-day round each. The mean fork length of all individuals was calculated within each group and varied between 107-167 mm (Fig. 1).

Figure 1. The mean fork length of the groups of 6 juvenile brown trout divided into similar sizes within each section and round of the artificial stream Hitec Flume.

| Round | Section | Mean fork length (mm) |
|---|---|---|
| **1** | 1 | 167 |
| **1** | 2 | 151 |
| **1** | 3 | 134 |
| **1** | 4 | 116 |
| **2** | 1 | 136 |
| **2** | 2 | 124 |
| **2** | 3 | 105 |
| **2** | 4 | 116 |
| **3** | 1 | 107 |
| **3** | 2 | 116 |
| **3** | 3 | 125 |
| **3** | 4 | 120 |
| **4** | 1 | 136 |
| **4** | 2 | 133 |
| **4** | 3 | 116 |
| **4** | 4 | 145 |

# Phase 1. Experimental design: Hitec Flume

The HyTec Flumes are two 60-meter gravel bottom channels simulating the natural habitat of a small mountainous stream, resembling smaller pools where groups of juvenile brown trout would be restrained to in natural settings (Fig 2). The water is connected from a natural water stream nearby. Unlike the natural stream, HyTec Flumes allow for manipulation of water discharge and are equipped with an array of video cameras mounted above the sections, while Radio-Frequency - ID (DRIF) antennas are submerged in the bottom of the substrate for the registration of fish with PIT-tag passing through. The flume allowed for analysis of fish behavior in their natural habitat without disturbance from observers. The channels in the HyTec flumes were split into eight equivalent sections (N= 6), where the experimental fish were hosted. Each section is 7 m long, and 1.5 m wide and divided by a 2 m long buffer zone. The stream sections were divided into three different habitat qualities with different kinds of substrate and amount of dead wood, with the lowest quality furthest downstream, intermediate quality in the middle section, and the habitat with the highest quality furthest upstream with a possibility for shelter in each quality (Fig. 3). The water temperature naturally varied between 8.5 – 9.4 °C at the deep section and 12 -14.7 °C at the surface. The water level was manually manipulated with wooden boards between the sections and lowered on day 6 of each round before increasing on day 7 to investigate the effect of different water levels, and therefore also more space for the fish on the social hierarchy and aggression. On day 7 of the rounds, one individual from each section was removed to investigate the stability of the social hierarchy, where one individual was selected that was taking shelter at the highest quality habitat during telemetry.

Figure 2. Hitec Flume with 8 sections divided into two artificial streams located in Lunz am see, Austria. Photo: Libor Závorka

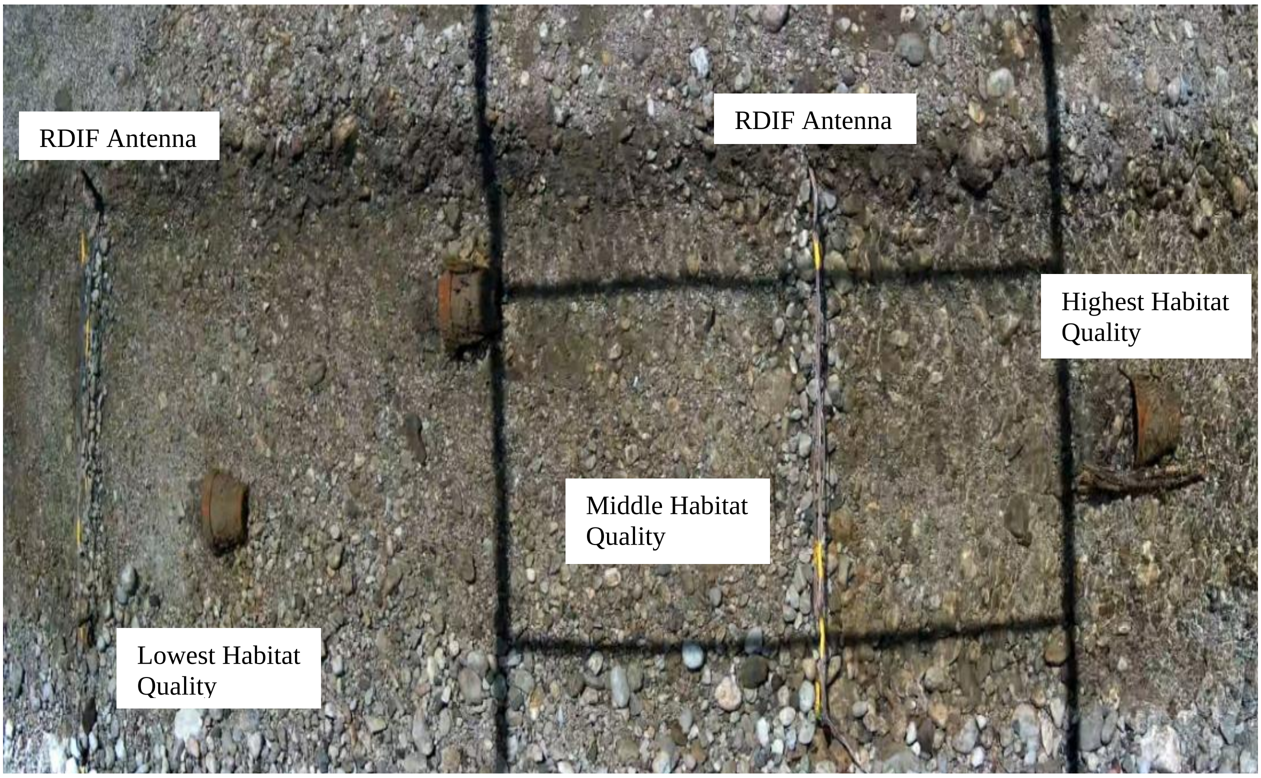

Figure 3. Hitec flume with three different quality of habitats, highest quality upstream that include the highest complexity of structure, middle quality with less structure and lowest quality downstream with no structure. RDIF antenna upstream and downstream

## Video analysis

The examination of the behaviors of the juvenile brown trout in Hitec flume was carried out through video analysis of the 25 min videos of the flume divided into 5-minute sections and the number of attacks, attacked individals and the presence of an established individual was categorized along with the time of dominant individual passing through RDIF antennas for identification. The identification of the dominant fish was made by analyzing boldness, aggressiveness, and activity where the dominant individual was observed guarding the upper part of the stream, had a higher level of aggression towards the other group members in combination with the observed lighter colorations after competition, which was visible on video analysis.

Each group (section) was used in the experiment over one 10-day round, where four rounds were conducted in total between 16/6- 28/8-2023. In two of the rounds, four sections were used while in the other two rounds three sections were instead used. There were four levels (days of rounds) of the experimental round that were analyzed: 3 (2/3), 6 (5/6), 8 (7/8) and 10 (9/10) meaning that it started with time interval (time of day) 15 on day 2, continued with time interval 21 at the same day and finished with interval 01 and 05 on day 3 and so on through the days of round. During time interval 01 the established dominance was not checked due to limited vision at nighttime, meaning that there were difficulties identifying all individuals from their dominant or subordinate colorations. The days have been divided to include the different parts of the experimental round:

- Day 3: Initial stage of the social dynamic
- Day 6: Social dynamic under low water conditions
- Day 8: Social dynamics after removal of one individual from the group.
- Day 10: Final stage of the social dynamic

## Phase 2. Experimental design: Sex identification

To receive information of the sex of the established dominant individual of the different sections in the Hitec flumes, the PIT-tag number of the individual going through the RDIF antennas at the noted time from video analysis was paired with the sex of the same individual. The identification of sex was performed by extracting DNA for PCR and identification of the Y-chromosome gene (sdY) using fin clips (1-2 mm) in a molecular non-invasive method, which was used on all 96 juvenile brown trout in this study. This is a technique based on Meeker et al (2007) for “HOTSHOT” genomic DNA extractions. The extracted DNA was later used for sex identification with PCR, based on Yano et al (2012) and Quémére et al (2014). For a detailed description of DNA extraction and sex identification with PCR, see Addendum 2.

## Statistical analysis

Multiple linear regression was used to analyze the combination of behavior and sex using data from video analysis during the different 10-day experimental rounds at the Hitec Flume and the sex identification from DNA of the same test organisms. It analyzed the relationship between:

- The residency (day of round), time of day and the number of attacks and attacked individuals.
- The quota of visible established dominance per round at different time of day and residency.
- Sex of dominant individual and number of attacks and attacked individuals.

These variables were also analyzed for significant statistical differences using type II ANOVA TEST. Graphs of the result will be included and show the chosen days observed to cover the different parts of the experimental round.

# Result

## Residency and time of day

The interaction between residency, time of day and the number of attacks and number of attacked individuals within the groups are both significant ($p=0.03$, $p<0.001$), with the highest levels at 5 am at the start of the rounds (Fig. 4 & 5).

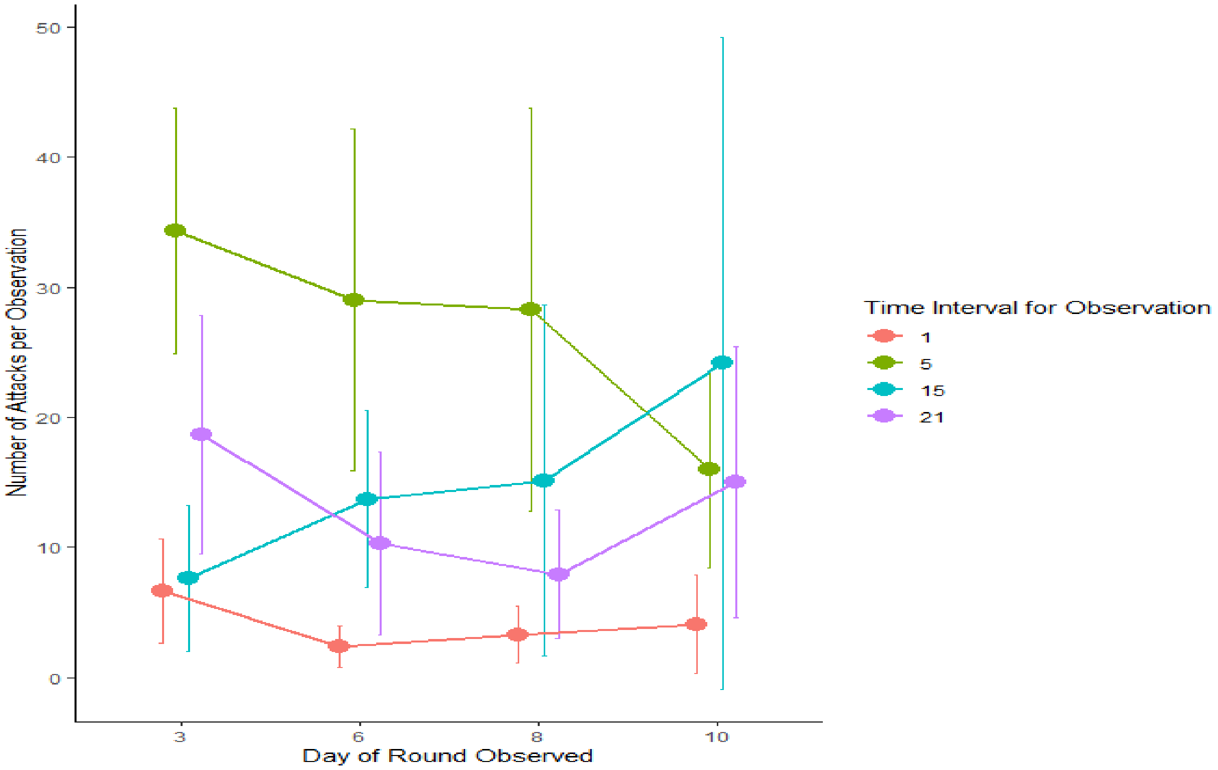

Figure 4. Linear regression model of the relationship between number or attacks during observation, day of round and time interval of observation between groups of juvenile brown trout in artificial stream Hitec flume (p=0.012). Adjusted R-squared=0. 3327, F= 2.1066, DF= 9. Error bars represent confidence interval (CI) of the mean.

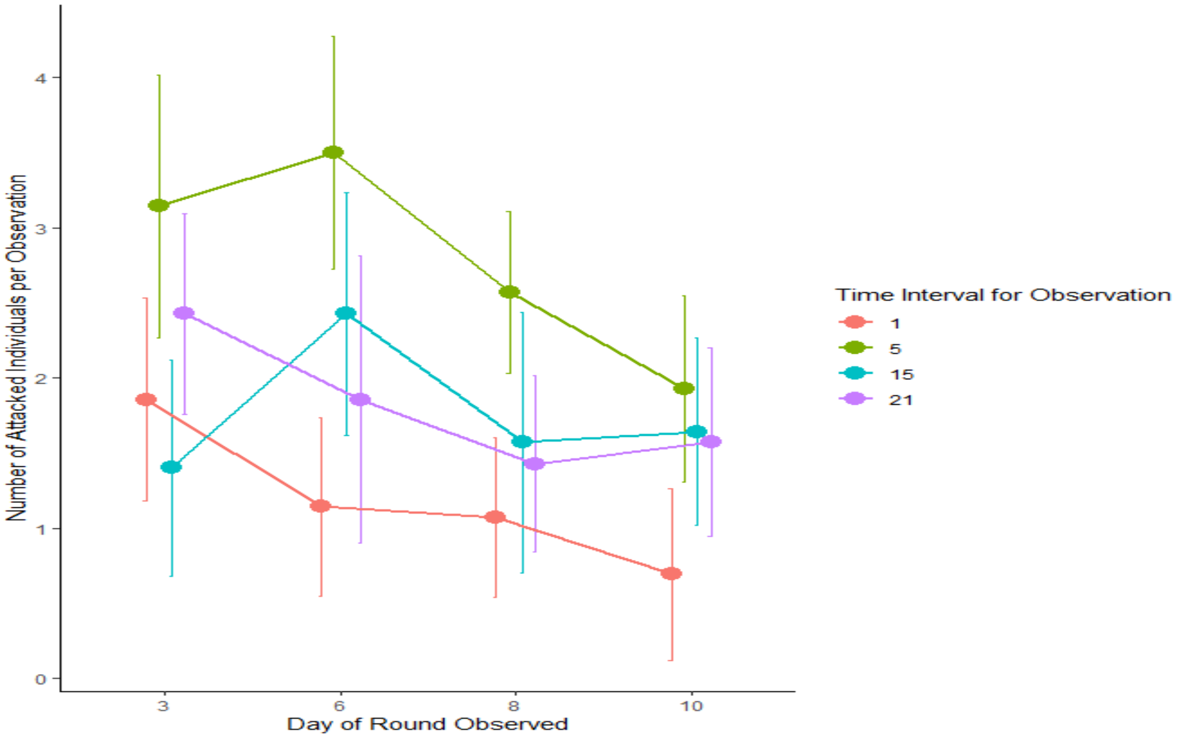

Figure 5. Linear regression model of the relationship between number of attacked individuals during observation, day of round (p<0.001) and time interval (p<0.001) of observation between groups of juvenile brown trout in artificial stream Hitec flume. F=5.91 (day of round observed), 17.299 (time interval for observation), DF= 3, 3. Error bars represent confidence interval (CI) of the mean values.

## Establishment of dominance

The interaction between establishment of dominance, day of round and time interval is significant (p<0.001), with an early higher visibly established dominance at 5 am and the highest number of visibly established dominances from day 8 throughout all time intervals (Fig 6).

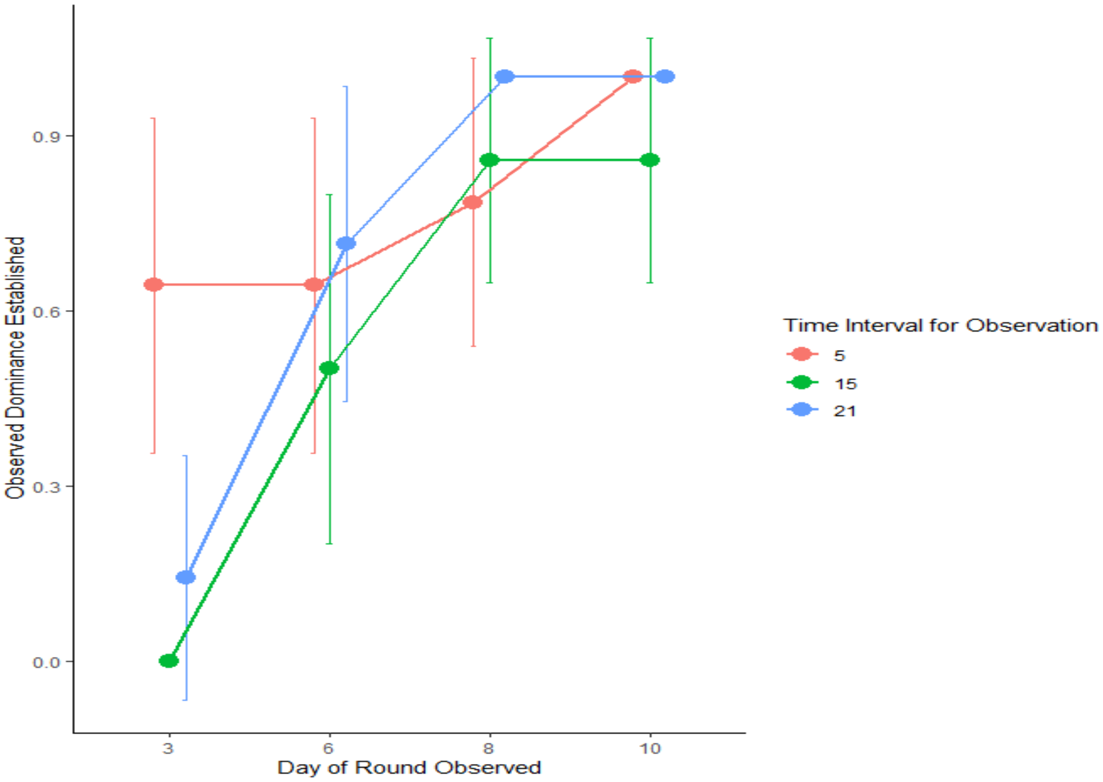

Figure 6. Multiple- linear regression model of the relationship between established dominance, day of round and time interval observed through video analysis of groups of juvenile brown trout in artificial stream Hitec flume (p<0.001). F=4.8964, DF= 6. Error bars representing the confidence interval (CI) of the mean value.

## Sex of dominance and aggression

The result concluded that the established dominant individuals from the video analyses were both males and females, whereas three sections had dominant individuals that could not be identified (Fig. 7). The interaction between the number of attacks and number of attacked individuals within the groups and sex of dominant is not significant (p=0.1808, p=0.089). However, when adding the factor of mean fork length in the interaction, the result is significant (p=0.012, p=<0.001), with an increase in attacks and attacked individuals with mean fork length and female dominant (Fig. 8 & 9).

Figure 7. Sex of dominant individual identified from video analyzing and sex identification within each section in respective round of the Hitec Flume experiment.

| ROUND | SECTION | SEX OF DOMINANT |
|---|---|---|
| 1 | 1 | Female |
| 1 | 2 | Male |
| 1 | 3 | Female |
| 2 | 1 | Male |
| 2 | 2 | Female |
| 2 | 3 | Female |
| 2 | 4 | Male |
| 3 | 1 | Female |
| 3 | 2 | Male |
| 3 | 3 | NA |
| 3 | 4 | NA |
| 4 | 1 | Male |
| 4 | 2 | Male |
| 4 | 3 | Male |
| 4 | 4 | NA |

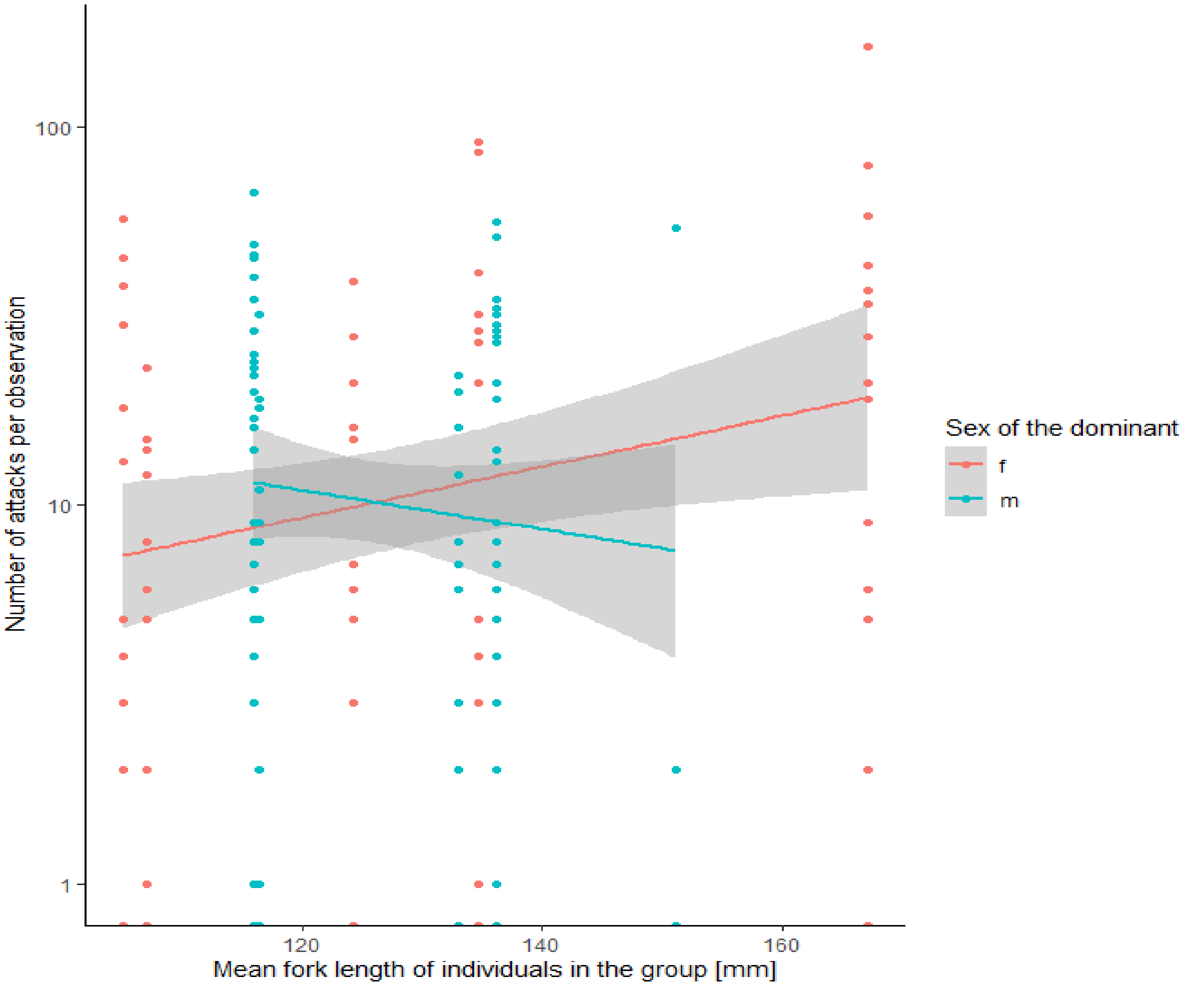

Figure 8. Linear regression model of the relationship between number of attacks during observation, mean fork length of individuals in the group (mm) and sex of the established dominant individual in each section in artificial stream Hitec flume with groups of juvenile brown trout. (p=0.012). Adjusted R-squared: 0.053. F=6.5030, DF= 1. Error bars represent confidence interval error bars (CI) of the mean value.

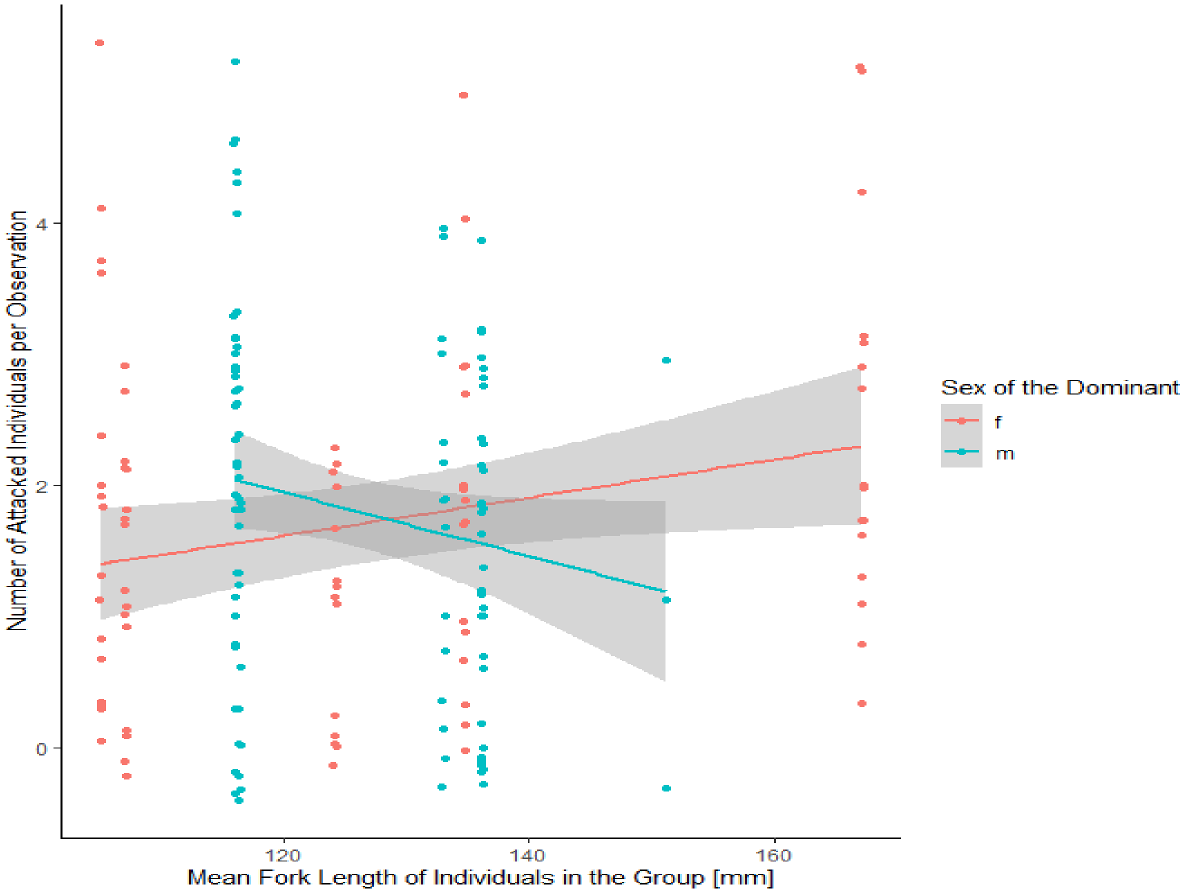

Figure 9. Linear regression model of the relationship between number of attacked individual during observation, mean fork length of individuals in the group (mm) and sex of the established dominant individual in each section in artificial stream Hitec flume with groups of juvenile brown trout. ($p<0.001$). F=19.7996, DF= 1. Error bars represent confidence interval (CI) of the mean value.

# Discussion

This study hypothesized that both attacks and number of attacked individuals would be significantly affected by the day of round and time of day and that there would be a significantly higher number of attacks and attacked individuals at dawn. This hypothesis was shown to align with the result from the Hitec Flumes. This result also coincided with earlier studies on salmonids claiming that there would be a significantly higher level of aggression at dawn after a period of darkness on a population level. The result of this study, however, does not coincide with earlier claims that there would be a significantly higher aggression level in the daytime, but partly coincides with the same study regarding the overall low aggression levels at nighttime. The study also hypothesized a trend in aggression in relation to the different social dynamic stages and the hypothesized establishment of dominant roles within the group. The result of this study was shown not completely aligned with this hypothesized aggression trend but instead partly aligned during different times of day. At the initial stage of the social dynamics (day 3), the aggression levels where significantly higher at dusk (5 am) with the second highest aggression level at dawn (9pm) at the start of the study. Nevertheless, at the social dynamic under low water conditions (day 6), the aggression level at 3 pm (daytime) surpasses the levels at dawn, coinciding with earlier studies claiming a positive relationship between aggression levels and number of days spent in the same place. This could signal that at daytime, the decrease in available area from the lower water levels leads to more aggressive interactions in the middle of day, even though they are more visible to predators in and out of the water. This could indicate that even though they were more visible to predators, the gain of better habitat and dominance from intraspecific competition was more worth energy expenditures and risk exposure than hiding from predators in the same confinement, similarly to pools close to their hatching sites in natural conditions.

The interaction between day of round and time of day also significantly affected the visibility of dominance establishment seen through video analysis. Dominant behaviour within the groups was more visible at dawn and stabilized across all sections and time intervals by day 8, confirming the hypothesis of the study. This early establishment of dominance at dawn coincided with a decrease in aggression at the same time in relation to the number of days in confinement, likely due to the order imposed by dominant individuals. Daytime instead showed increased aggression, correlating with a later establishment of dominance and indicates a lack of social order. However, from day 8, dominance was established even during the day, though aggression remained high. This suggests that newly established dominance may not be fully recognized, leading to ongoing competition. The persistent aggression despite dominance establishment might also indicate that dominant roles are less influential during the day, possibly because the fish became bolder over time. This increased daytime aggression aligns with studies suggesting higher activity during daylight. Additionally, the pattern of aggression did not follow the hypothesized trend except for the number of attacked individuals during the day. Fewer individuals faced more attacks post-dominance establishment, suggesting that dominant fish targeted specific subordinates challenging the hierarchy, especially at daytime.

There was no significant difference between body size and aggression which did not coincide with earlier studies, but when adding the factor of sex of the dominant there was a clear significance. While both sexes became dominant during the experiment, groups with female dominant exhibited a significantly higher number of attacks and attacked individuals with a higher group body size. In contrast, male-dominant groups maintained a steady number of attacks across different groups of body sizes, with a decrease in the number of attacked individuals as body size increased. The result might suggest that females are generally more aggressive when the area becomes more crowded due to increased group body size, leading to more attacks on more individuals compared to groups with male dominants. The higher aggression level in female-dominant groups with increased group size might be linked to the selection pressure on female body associated with fecundity, even before sexual maturation. Since growth rate is shown to be positively correlated with both higher fecundity in females, aggression, and survival at first year, larger females might experience selection pressure to establish dominance already at a juvenile stage, securing better territories with better feeding opportunities. The result of this study regarding sex of dominance and aggression aligns with earlier studies showing a positive correlation between growth rate and aggression. However, it contradicts studies that males are more consistently aggressive. While earlier studies have claimed that male juvenile salmonids are significantly more aggressive in interspecific interactions, they also showed that the most dominant individual may not consistently be male due to the insignificantly number of wins between the sexes. This partly coincides with the findings of this study, since both sexes was identified as the most dominant in the different groups, it did not coincide with the earlier claim that males are more aggressive. To be able to draw any conclusions, more studies about the subject involving more factors, a greater number of fish and longer rounds would be needed.

# Verification of methods

At the beginning of video analysis, the initial result was verified for accuracy by having five different analysts analyzing the same videos. The similarity of their results was checked using multiple linear regression, which showed no significant differences between the analyzes. Dissections were also performed on 30 of the 96 juvenile brown trout from the experiments to determine the accuracy of DNA identification against traditional manual dissection of sexual gametes. Out of the 30 individuals, one had mismatched sexes between the methods and another one had missing DNA in one of the samples from a fish that was dissection, meaning that 28 out of 30 showed the same sex when comparing the two methods. The mismatching and missing of DNA in sample could be due to contamination.

# Other

The high increase in the number of daytime attacks at the end of the rounds, and the lower nighttime attacks throughout the rounds, could be due to better visibility during video analysis at that time of day compared to other time intervals. In the Hitec flume, the fish were divided into similar sizes and trapped in one section per group which might have affected the overall behavior and social hierarchy, whereas in natural settings other individuals of greater body size would have become dominant. In natural settings with different size distributions, juveniles might relocate to other areas, but since their swimming capacity is limited at an early age and the Hitec Flume is designed to mimic a natural pool, it is difficult to conclude how much the residence in the artificial stream has affected their behavior. According to the graphs, the removal of an individual from a higher quality habitat on day 8 does not seem to have an overall effect on the aggression level throughout the time intervals, except for aggression at dawn where it was seen to drastically decrease after that day, and at daytime where aggression instead was drastically increased. This could entail that the removal of an individual that was residing in the higher quality habitat during telemetry might have changed the dynamic of the groups, but since there is an establishment occurring throughout the different rounds, the removed individual might not have been the most dominant after all. The social dynamics under low water conditions do not seem to significantly affect the aggression level according to the graphs either. However, the number of attacked individuals does show a spike on the same day, which could be due to the lower water conditions, since smaller areas might lead to a higher number of attacked individuals when the possible hiding places shrink. The antennas used in the Hitec Flume were not always working, leading to only 15 sections and 90 fish being statistically analyzed instead of the original 96 fish and 16 sections. The sex ratio of the different groups was not controlled for in the Hitec Flume experiment, later analysis revealed that when one sex predominated in a group, one individual of that sex tended to become dominant. In groups with an equal ratio of males and females, both sexes displayed dominance behavior in equal number of sections. This shows that sex of dominant can represent both sex ratio of the groups as well as the sex of the most dominant individual. However, the sex ratio was not factored into statistical analysis due to its strong correlation with dominant sex, which could lead to inaccurate results.

# Conclusion

This study validates the importance of high-quality, complex juvenile salmonid habitats by illustrating how social hierarchies can establish dominance and maintain order within different quality micro-habitats. These social structures are needed for a sustainable population, indicating the occurrence of different levels of aggression and establishment of dominance in habitats with different structures and complexity. It also showed that both time of day and how long juveniles had been confined in the same area, similar to natural conditions, had a significant effect on the aggression level within the whole group, with fluctuations during the different time intervals and a significantly highest level of aggression occurring at dawn. This study also showed a relationship between the establishment of dominance, time of day and days of confinement, where the establishment of dominance was visible at an early stage at dawn and throughout all groups from day 8. The result of this study also reveals how sex and body size contribute to dominance and aggression levels, with the key findings of groups with female dominants were more aggressive with a higher group body size, likely due to female juveniles early need to allocate resources to increase body size and improve fecundity.

The result of this study may help predict resources distribution within fish populations and help understanding of the need of salmonids when restoring important stream sections for reproduction and shallow areas for residence during juvenile stages to increase their likelihood of surviving to sexually mature adults. The method of sex identification may help for faster analysis of sex ratio in streams, which might also show the migratory properties of the location, since females (around 10 cm fork length) are known to migrate earlier than males (Lavender et al., 2023). The findings of this study may also enlighten the cultivation of juvenile salmonids in hatchery, ensuring they develop the necessary social skills and behaviors before being released into the wild. This may also increase their chances of survival. By contributing to the broader understanding of salmonid biology and ecology, the study supports preserving biodiversity.

# Future studies

To further develop dominance assessments using juvenile salmonids, a study that mainly focused on behavior of sex on an individual level should divide the groups into specific ratios, which will result in a more controlled response in the group dynamics. To get more secure data on the different temporal and dynamic factors on a population level, the experimental rounds should be longer, with the use of more individuals. The time of year for the experiment can significantly impact on the behavioral aspects of the study. According to research on the annual behavioral patterns of salmonids, these fish are more diurnal during the summer months when water temperature consistently exceeds around 10 degrees Celsius. During winter months, they instead become more nocturnal when the temperature decreases. This alteration is driven by changes in the retina of the fish, which is temperature-dependent and adapts to darker surroundings and enhances their vision in low-light conditions (Fraser et al., 1993). Another study of the subject of temperature and dominance in juvenile salmonids could conclude that salmonids in colder temperatures were instead more dominant than salmonids in warmer temperature (Leksell, 2017). To gain a deeper understanding of social behaviors during winter, it would be beneficial to combine a short-assessment of sex-specific behavior at both individual and populations level in juvenile salmonids with data collected during winter, with more factors included, such as feeding attempts, visible individuals and the tracking of different choices of habitats throughout multiple days, not only in dominant individuals. This may be conducted over a longer period with a larger sample size. This may benefit the overall conservation work during the colder months, but also to gain further knowledge of how salmonids will adapt to climate change and warmer weather, specifically during winter months. Employing non-lethal sex identification methods in such studies would be particularly advantageous and further dominance studies conducted in natural settings that includes fish captured from the same stream might also be preferably, to make the experiment as natural-like as possible.

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# Addendum 1.
## Popular science summary

## Are male or female juvenile brown trout more aggressive and dominant?

New research shows that both sexes of juvenile brown trout are dominant and that females with bigger body size are more aggressive than males, which goes against earlier studies.

Juvenile brown trout. Foto: Jorge R Sánchez-González

### Why study salmonid species and the behaviors between sexes of juveniles?

Salmonids are of great interest to researchers due to their complex group structures and survival strategies. Much behavioral research focuses on adults since their sex can be visibly identified. However, in juveniles, which look identical before sexual maturation, this is not possible, leading to a research gap in sex-related behavior. Traditionally, sex identification in juveniles required dissection, but to conserve these fish, non-lethal methods are needed.

**Earlier studies**

Earlier studies shows that adult male salmonids are more dominant and aggressive. Research on juvenile brown trout and rainbow trout also indicates that males are more aggressive.

### The aim of this study

The aim of the study was to analyze if aggression is connected to the number of days the fish have been in the same place and the time of day and if establishment of dominance is visible within the 10-day rounds and higher at a certain time of the day.

The aim of the study was also to determine whether male or female brown trout are more dominant and aggressive during their juvenile stage and if that also can be connected to the size of the fish in the group.

## Method

In this study, a new molecular technique was used to identify the presence of the sdY gene in 186 juvenile brown trout, which is a gene only found in males. This was done by extracting DNA from a small fin piece (1 – 2 mm). This method keeps the fish alive and unharmed.

The same fish were then placed in an artificial water stream “Hitec flume” divided into sections with different habitat qualities, where aggression and dominance were observed through video analysis over 10-day rounds at different times of the day (Fig. 1).

Figure 1. Artificial water stream "Hitec Flume" divided into eight sections with different habitat qualities. Photo: Libor Závorka

## Result

The study showed that number of attacks, attacked individuals and establishment of dominance are all dependent on day of round and time of day, and was mostly visible at 5 am but became more overall visible at all hours of the day after day 8. A higher number of attacks and attacked individuals was visible at 5 am every day but decreases throughout the rounds. The decrease in the overall aggression could be due to early visible establishment of dominance visible at the same time, since dominance is shown to decrease aggression and bring order to the social hierarchy of the groups. The level of aggression visible at 15:00 (3 pm) instead increased throughout the round and could be due to late dominance establishment at the same time of day, since duration of time spent in one place is shown to increase aggression. From day 8 or the rounds, dominance was visible at all times of the day, all agreeing fully or partly with earlier studies.

Both sexes became dominant, but neither sex was more aggressive overall. However, females showed increased aggression with rising body size, which goes against earlier studies, and may indicating higher reproductive pressure to establish good habitats early when females are of bigger size, unlike males, who may face this increased pressure only after sexual maturation.

# Addendum 2
## Chemicals and methods

### HOTSHOT's Genomic DNA Extraction

The reagents used for extraction of DNA was alkaline lysis buffer**:** 50 mM NaOH (pH ~ 12.0) where NaOH stock solution was diluted for obtaining working solution. 250 µl of 10 N NaOH was added to 49.75 ml $ddH_2O$ for a final concentration of 50 mM NaOH. No pH adjustment required. The other reagent used in the extraction was neutralizing Buffer: 1M Tris-HCl (pH ~ 8).

**Alkaline Lysis buffer (sodium hydroxide NaOH):**
**For 100 mL stock 10 M NaOH:**

1. 40 g NaOH (molecular weight: 40)
2. Dissolve in 70 mL deionized/distilled water (a few pellets at a time)
3. Adjust volume to 100 mL with deionized/distilled water ($ddH_2O$)
4. Store at room temp

**Working solution (for HotShot extractions) 50 mM NaOH (pH ~ 12.0):**

1. Add 250 µL of 10 M NaOH stock to 49.75 mL $ddH_2O$
2. No pH adjustment necessary
3. Store at room temp (1–2 weeks only)

**Neutralizing buffer (1 M Tris-HCl pH 8.0 solution):**

**To make 100 mL solution:**

1. Weigh 12.11 g of tris base and add to Duran bottle.
2. Measure 80 mL distilled water and add to Duran bottle.
3. Mix the solution.
4. Add pH meter to the solution to observe pH.
5. Slowly add concentrated hydrochloric acid (HCl) solution to reduce pH to 8.0.
6. Once desired pH is reached, top up solution to 100 mL using distilled water.
7. Autoclave
8. Store at room temperature

**Method**

Small tissue sample (e.g. fin clips) of around 1-2 mm was placed into a PCR tube with 30 µL of 50 mM NaOH (Alkaline buffer), the samples were completely covered. The samples were then heated up to 95°C for 15-30 minutes (less time for very small or very soft tissues) in a thermocycler. After held at 4°C to cool sample. 30 µL of 1M Tris-HCl (Neutralizing buffer) (pH~8.0) was added to the tissue to neutralize the solution and mixed. The sample was centrifuged for 10 minutes at 5000 u to pellet the tissue debris. Everything was left in the same tube. The supernatant can be directly used for PCR reactions. 1–5 µL of supernatant was used for the PCR. The DNA can be stored at 4°C for up to 3 months or at –20°C for long-term storage.

### Sexing with PCR

The DNA extracted using the "HOTSHOT" Genomic DNA Extraction method was later used in the sex identification of the sdY gene using PCR.

**Primers**

The primers Salmo18-S and Salmo sdY were used for positive amplification control (Fig. 2).

Figure 2. Primers and sequences used in sex identification of juvenile brown trout with fin clips.

| **Primer name** | **Sequence (5'-3')** |
|---|---|
| Salmo sdY-Forward (primer F1) | CCC AGC ACT GTT TTC TTG TCT CA |
| Salmo sdY-Reverse (primer R1) | CTT AAA ACC ACT CCA CCC TCC AT |

| | |
|---|---|
| Salmo 18S-Forward (primer F2) | GTY CGA AGA CGA TCA GAT ACC GT |
| Salmo 18S-Reverse (primer R2) | CCG CAT AAC TAG TTA GCA TGC CG |

## Master mix PCR

Regents and their components of master mix for PCR (Fig 3 & 4).

Figure 3. Reagents and their specific concentrations for master mix used for sex identification of salmonids using DNA from fork clips.

| Reagent | Conc. | Final Conc | 1 unit | 74 samples |
|---|---|---|---|---|
| H20 | | | 2.75µl | 203,5µl |
| Green buffer | 5x | 1x | 2µl | 148µl |
| MgCI2 (vortex before use) | 25mM | 1mM | 2µl | 148µl |
| Primer F1 | 10µM/µl | 0.5µM | 0.5µl | 37µl |
| Primer R1 | 10µM/µl | 0.5µM | 0.5µl | 37µl |
| Primer F2 | 10µM/µl 0 | 0.5µM | 0.5µl | 37µl |
| Primer R2 | 10µM/µl | 0.5µM | 0.5µl | 37µl |
| dNTPs mix | 10µM/µl | 0.2 mM | 0.2µl | 14.8µl |
| GoTaq | 5 U/10µl | 1.25 U | 0.05µl | 3.7µl |
| DNA (not included in master mix) | | | 1µl | 74µl |
| Final volume | | 10µl | 10µl | 740µl |

Figure 4. Components for master mix and their final volume and concentration used for sex identification of salmonids using DNA from fork clips.

| Component | Final Volume | Final Concentration |
|---|---|---|
| 5x Green or colorless GoTaq ® Flexi buffer [1] | 10 µl | 1 X |
| $MgCl_2$ Solution 25mM[1] | 2-8 µl | 1.0-4.0 mM |
| PCR Nucleotide Mix, 10mM each | 1 µl | 0.1-1.0 µM |
| Upstream primer | X µl | 0.1-1.0 µM |
| Downstream primer | Y µl | 0.1-1.0 µM |
| GoTaq ® G2 Hot Start Polymerase (5u/ µl) | 0.25 µl | 1.25 u |
| Template DNA | Z µl | <0.5 µg/50 µl |

### dNTPs:

for 50 µl master mix 1 µl dNTPs is recommended

### Polymerase:

For 50 µl master mix 0.25 µl is recommended
C1= 5u µl, c2=final con. U/final vol=U/ µl
V1=x, V2=final vol

### MgCI2:

C1= 4 mM, C2= 8µl
V1= 1 mM, V2= Xµl, x=1*8/4

## Cycling conditions for Gel 1

Settings used in the PCR for sex identification (Fig 5).

Figure 5. Settings used in the PCR for sex identification, including the different steps, temperatures, time, and cycles for sexing of brown trout with fin clips.

| Stage of cycling condition | Temp °C | Time | Cycles |
|---|---|---|---|
| Denat.Initial | 95 | 15 mins | |
| Denaturation | 94 | 30 sec | |
| Annealing | 63 | 45 sec | |

|  |  |  |  |
|---|---|---|---|
| Elongation | 72 | 30 sec | 2x 35x |
| Elongating final | 72 | 5 min |  |
| Cooling | 4 |  |  |

**Visualization**

Ingredients used for visualization of one or two bands:

1.5% agarose gel with SybrSafe dye (e.g. 1.5 g agarose, 120 mL TBE, 10 µL SybrSafe), 3 µL of PegGreen for 30 min. 3 µl/Slot template, 100 V, 1 kb Thermo scientific geneRuler DNA ladder, ready-to-use. The gel electrophoresis was run at 100 V for 30–40 mins. Males were visualized with two bands (18S and sdY), while females were visualized with only one band (18S only) after PCR (Fig. 6).

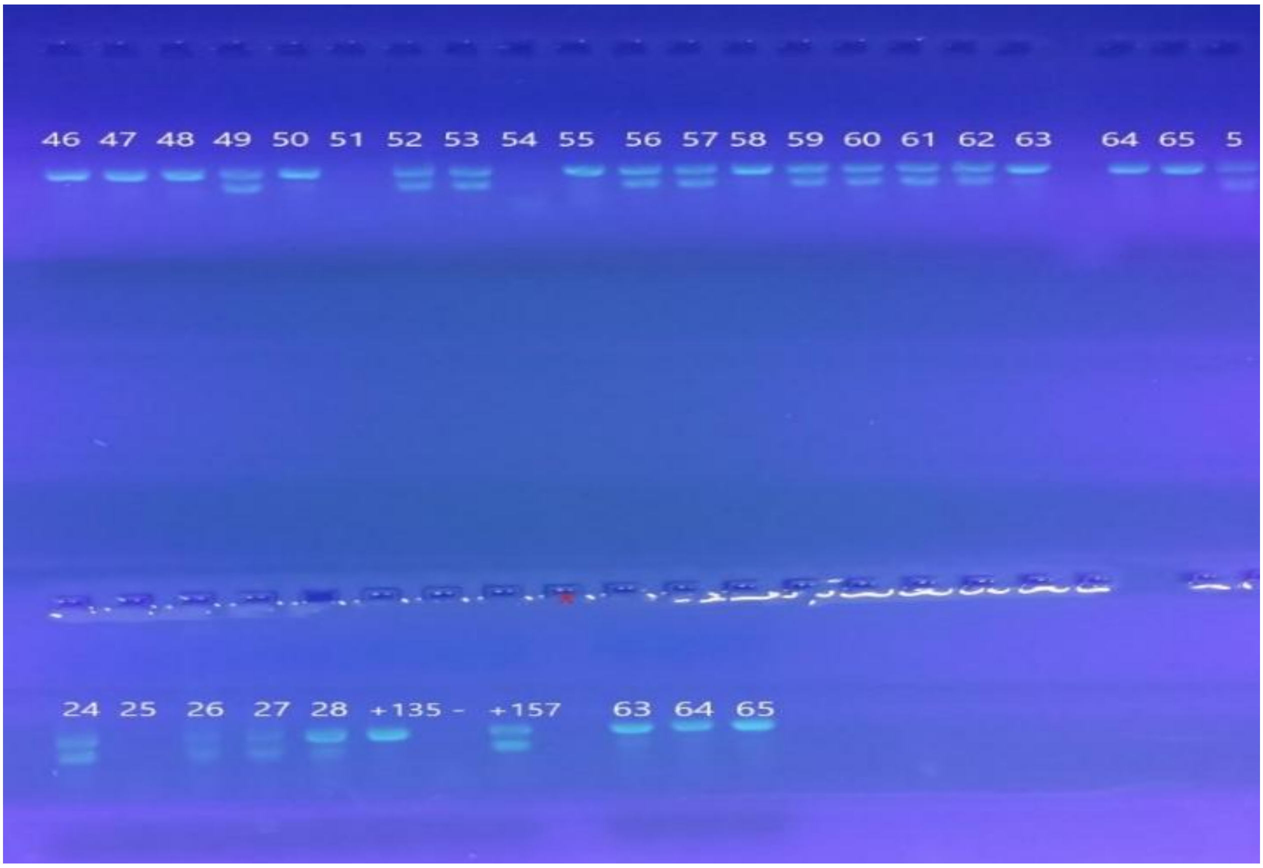

Figure 6. 96 brown trout sexed visually with gel electrophoresis after PCR analysis. Males have two bands (sdY and 18S) whereas females only have one band (i.e. 18S only). Photo: Theresa Reichenpfader.