

1 **Proliferative kidney disease in brown trout – infection level, pathology and mortality under field**
2 **conditions**

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10 Running title: PKD related mortality and pathology under field conditions

11

12 **ABSTRACT:**

13 Proliferative kidney disease (PKD) is an emerging disease threatening wild salmonid populations. In
14 temperature controlled aquaria, PKD can cause mortality rates of up to 85% in rainbow trout. So far,
15 no data about PKD related mortality in wild brown trout are available. The aim of this study was to
16 investigate mortality rates and pathology in brown trout kept in a cage within a natural river habitat
17 which is known to harbor *Tetracapsuloides bryosalmonae*. Young of the year (YOY) brown trout, free
18 of *T. bryosalmonae*, were exposed in the river Wutach, in the Northeast of Switzerland, during three
19 summer months. Samples of wild brown trout caught by electrofishing near the cage location were
20 examined in parallel. The incidence of PKD in cage exposed animals (69%) was not significantly
21 different to the disease prevalence of wild fish, 82 and 80%. The mortality in cage exposed animals,
22 however, was as low as 15%. At the termination of the exposure experiment, surviving fish showed
23 histological lesions typical for PKD regression, suggesting that many YOY brown trout survive the
24 initial infection. Our results at the river Wutach suggest that PKD in brown trout does not always
25 result in high mortality under natural conditions.

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27 KEY WORDS: proliferative kidney disease • brown trout • mortality • pathology • temperature

28

29 INTRODUCTION

30 Proliferative kidney disease (PKD) is an emerging disease of wild and farmed salmonid fish in Europe
31 (Okamura et al. 2011). The disease is caused by the malacosporean parasite *Tetracapsuloides*
32 *bryosalmonae* belonging to the Myxozoa (Hedrick et al. 1993, Canning et al. 2000, Okamura et al.
33 2001). The parasite's complex life cycle involves bryozoans as invertebrate (Anderson et al. 1999,
34 Longshaw et al. 1999, Okamura et al. 2001) and salmonids as vertebrate hosts (Feist & Bucke 1993,
35 Hedrick et al. 1993). *T. bryosalmonae* infects salmonids through skin and gills (Feist et al. 2001,
36 Longshaw et al. 2002) or gills only (Morris et al. 2000, Grabner & El-Matbouli 2010), and is
37 afterwards distributed systemically. The main target organ in the fish host is the kidney (Kent &
38 Hedrick 1985), where it multiplies and differentiates from extrasporogonic stages in the renal
39 interstitium to sporogonic stages in the lumen of renal tubuli (Kent & Hedrick 1985). Spores are then
40 excreted via the urinary system (Kent & Hedrick 1985, Morris et al. 2002, Hedrick et al. 2004, Bettge
41 et al. 2009). Transmission to bryozoans has been demonstrated in brown and brook trout (Morris &
42 Adams 2006, Grabner & El - Matbouli 2008). In affected young-of-the-year (YOY) salmonids,
43 proliferative and granulomatous nephritis and necrotizing vasculitis with thrombus formation has been
44 described (Hedrick et al. 1993, El-Matbouli & Hoffmann 1994, Bettge et al. 2009). Surviving fish
45 show chronic lesions with interstitial fibrosis and tubulonephrosis, followed by a complete
46 regeneration of renal morphology (Schmidt-Posthaus et al. 2012, 2013). The spread and outcome of
47 the infection are suspected to be enhanced by water temperature with severe disease and increased
48 mortality at temperatures above 15°C (Wahli et al. 2008, Bettge et al. 2009, Okamura et al. 2011). In
49 temperature controlled aquaria, PKD associated pathology and mortality are well documented in
50 rainbow trout (*Oncorhynchus mykiss*, Bettge et al. 2009, Schmidt-Posthaus et al. 2012). These studies
51 showed mortality up to 85% at a constant water temperature of 18°C. It was hypothesized that
52 infection with *T. bryosalmonae* plays a significant role in the decline of wild salmonid populations,
53 e.g. brown trout (*Salmo trutta fario*) in Switzerland and wild Atlantic salmon (*Salmo salar*) in Central

54 Norway (Wahli et al. 2002, Sterud et al. 2007, Krkošek et al. 2009). A widespread distribution of the
55 disease in wild salmonid populations was also shown in other countries (Dash and Vasemägi 2014,
56 Feist et al. 2002, Skovgaard and Buchmann 2012). However, so far, these studies only investigated
57 prevalence, infection intensity and associated pathology under field conditions. Mortality rates and
58 causes of mortality or contributing factors to it were not evaluated and are still under debate (Dash and
59 Vasemägi 2014, Skovgaard and Buchmann 2012).

60 The aim of this study was to investigate PKD-related pathology and mortality in YOY brown trout
61 exposed in a cage experiment to river water within their natural environment. Specifically, the
62 following questions were investigated: (i) Does infection with *T. bryosalmonae* cause mortality in
63 YOY brown trout under fluctuating temperature conditions as seen in the field?; and (ii) Does
64 infection with *T. bryosalmonae* cause comparable pathology in cage exposed brown trout as seen in
65 wild caught animals?

66

67 **MATERIAL AND METHODS**

68 **Study sites and fish sampling**

69 Experiments were conducted over a period of 3 months (16th of July to 8th of October 2013). The
70 examined river system (Wutach) is situated in the southern part of Germany and has a 4.8 km border
71 section to Switzerland in the midsection of the river. It passes through rural and urban areas before
72 feeding into the larger river Rhine (Fig. 1). The Wutach has several tributaries; one of these was
73 included in this study (Ehrenbach) (Fig. 1). Fish can migrate from the tributary into the Wutach but
74 not vice versa because of an unpassable barrier. In former years, brown trout were regularly stocked in
75 the Wutach (stocked fish originated from a trout hatchery nearby), but catches by anglers and
76 electrofishing resulted in decreased numbers of fish in the midsection of the river (fishing statistics
77 1979-2013, unpublished data). Stocking was stopped one year prior to the beginning of the
78 experiment. Therefore, investigated animals from the field sampling originated from natural spawning.
79 In the tributary (Ehrenbach) included in this study, a stable brown trout population was recorded
80 (fishing statistics 1979-2013, unpublished data) and no PKD records exist.

81 Investigations were conducted in two separate approaches, (i) a cage experiment was performed
82 exposing YOY brown trout for three months to water in the Wutach; (ii) wild brown trout were
83 sampled by electrofishing in two stretches in the Wutach and at one stretch in the Ehrenbach (field
84 sampling). Water temperature was measured inside the cage and at two locations in river water, in the
85 Wutach and the Ehrenbach.

86

87 *Cage experiment*

88 A cage, 1x1x0.5 m, was placed into the Wutach in the midstream part of the river (Fig. 1). Mesh size
89 of the cage was 1 cm in diameter. An additional mesh was placed outside of the cage in the upstream
90 position to avoid blocking of the cage by large floating refuses. This mesh was cleaned regularly.

91 During the experiment, the cage itself was not cleaned to avoid additional stress of the exposed
92 animals. 100 YOY brown trout were purchased from a trout hatchery. Offspring of various
93 broodstock, originally deriving from the Wutach, were mixed on the farm. Animals used in this
94 experiment therefore originated from various mother animals. To determine if stocked fish were
95 negative for infection with *T. bryosalmonae* at the beginning of the experiment, five brown trout were
96 euthanized in clove oil and immediately examined as described below. Ten brown trout served as a
97 negative control and were kept in spring water at the hatchery of origin.

98 In the middle of July 2013, 85 brown trout were placed into the cage. They were fed 1% of body
99 weight with commercial fish food (TroCo Ultra, Coppens, Netherlands, pellet size 2 mm) using an
100 automatic feeding machine ensuring continuous food supply. All fish were monitored daily for signs
101 of disease or mortality. Moribund or dead fish were removed and investigated immediately. Three
102 months after the start of the exposure experiment, all remaining trout were euthanized in clove oil,
103 tagged and immediately investigated.

104

105 *Field sampling*

106 Fish were sampled at two stretches in the river Wutach, one stretch 2 to 3 km upstream of the cage
107 experiment and one stretch 3 to 4 km downstream (Fig. 1). Additionally, the tributary was sampled a

108 few kilometres upstream of the entry into the river Wutach (Fig. 1). In September 2013, 10 and 11
109 YOY brown trout, respectively, were sampled at each of the two sites in the Wutach and 15 YOY
110 brown trout in the Ehrenbach by electrofishing. Due to low density of YOY it was not possible to
111 sample higher numbers of animals. Fish were euthanized separately in clove oil and examined as
112 described below.

113

114 **Pathology, histopathology and immunohistochemistry**

115 Length of every dead or euthanized fish was recorded, followed by a complete necropsy. Animals
116 originating from the cage experiment were weighted and the condition factor was calculated
117 ($100 \times \text{weight} / \text{length}^3$, Bagenal, 1978). Macroscopic changes in the inner organs were recorded.
118 Animals were then immediately fixed in 10 % buffered formalin for histopathological and
119 immunohistochemical (IHC) examination. Kidney samples of all animals (cage experiment, field
120 sampling) were routinely paraffin-embedded and 4 μm sections were prepared for haematoxylin-eosin
121 (H&E) stain. Suspicious cases were examined by IHC using a monoclonal anti-*Tetracapsuloides*
122 *bryosalmonae* (PKX) antibody (AquaMAb-P01, Aquatic Diagnostics Ltd., Stirling, UK, Adams et al.
123 1992). Histopathological changes of the kidney were graded according to a common pathological
124 grading system as 0 (no) to 6 (severe). Presence of *T. bryosalmonae* was examined on whole
125 histological and IHC kidney sections and infection intensity was classified as 0 (no parasites present
126 on whole slide), 1 (single parasites), 2 (mild infection rate), 3 (mild to moderate infection rate), 4
127 (moderate infection rate), 5 (moderate to severe infection rate) or 6 (severe infection rate) at a
128 magnification of 200 to 400x.

129

130 **Real time PCR for detection of *T. bryosalmonae* DNA in kidney tissue**

131 Fish negative for *T. bryosalmonae* by histology and / or IHC were examined by real time PCR. Two
132 20 μm sections of paraffin embedded material were deparaffinized. Lysis and extraction of total DNA
133 was performed using DNeasy tissue Kit (Quiagen, Basel, Switzerland) according to the manufacturer's
134 protocol. Samples were incubated with proteinase K at 56°C and 450 rpm overnight. The yield was

135 determined by spectrophotometry using the NanoDrop photometer (NanoDrop Technologies, Inc.,
136 Wilmington, USA). Real time PCR was performed using SYBR GoTaq® qPCR Master Mix
137 (Promega, Dübendorf, Switzerland) according to the manufacturer's instructions. Primer pair PKX3F
138 (CTAAGTACATACTTCGGTAGA) and PKX4R (CCGTTACAACCTTGTTAGGAA), described by
139 Kent et al. (1998), was used. A positive control sample obtained from the kidney of clinically infected
140 brown trout from other studies and a negative control using water were included in the PCR
141 procedure. A 297bp gene sequence of the small subunit ribosomal DNA (SSU rDNA) was detected.
142 Samples with a threshold cycle value (C_t) of 35 or lower and a melting temperature equal to the
143 positive control were classified as positive. To confirm the specificity of the real time PCR, the real
144 time PCR was repeated without dissociation stage and the PCR products were purified with
145 NucleoSpint® (Machery-Nagel, Oensingen, Switzerland). The products were checked on a 1%
146 agarose gel for amplification and molecular weight. To verify the sequence, the PCR products were
147 sent to Microsynth AG, Balgach, Switzerland for sequencing. The identity of PCR products was
148 determined by BLAST-n searching (Altschul et al, 1997) of the available sequences in the GenBank
149 database (www.ncbi.nlm.nih.gov).

150

151 **Water temperature**

152 Water temperature was recorded every 2 hours by temperature loggers located inside the cage, in the
153 Wutach near the cage, and in the Ehrenbach near the fish sampling site (Fig. 1). Mean values for each
154 day were calculated and reported.

155

156 **Statistics**

157 PKD prevalence was calculated as the sum of *T. bryosalmonae* positive animals per group divided by
158 the total number of animals per group (percentage). The incidence of PKD describes the percentage of
159 newly diseased individuals in the numerically defined group at risk for infestation over a limited time
160 period. Significant differences between the groups were tested using the chi-square-test at a $p \leq 0.05$
161 significance level.

162 Parasite infection intensity was calculated by the sum of values for infection intensity per fish divided
163 by the total number of infected animals. Mean of pathology score was calculated by the sum of
164 pathology scores per fish divided by the total number of affected animals.

165

166 **RESULTS**

167 **Water Temperature**

168 In the Wutach, water temperature constantly exceeded 15°C for 38 days in a row from July to August
169 (Fig. 1). Afterwards, water temperature exceeded 15°C over 10 additional non-consecutive days. As
170 fish in the cage were exposed starting in the middle of July, when water temperature was already high,
171 these animals experienced 39 days of water temperature above 15°C. In the Ehrenbach, water
172 temperatures of 15°C and higher were only reached on 8 days, and no longer than 5 days in a row.

173

174 **Cage experiment**

175 *Infection incidence, intensity, mortality and pathology*

176 Animals sampled at the beginning of the experiment before exposure to river water and those kept in
177 the hatchery were in good body condition, and showed no signs of *T. bryosalmonae* infection. No *T.*
178 *bryosalmonae* positive trout could be identified by real time PCR. No mortality was recorded in the
179 group kept at the fish farm.

180 Inside the cage in the river Wutach, 15 brown trout died during the first 73 days (Fig. 2). Two animals
181 which died during the first 14 days of the experiment showed neither macroscopical signs of PKD nor
182 any histological kidney pathology nor presence of *T. bryosalmonae* DNA. In all animals that died at
183 later time points, infection with *T. bryosalmonae* was evident. Parasites were visible histologically in
184 nine animals. These nine animals showed moderate to severe kidney lesions typical for an acute PKD
185 infection (mean value: 3.33, SD: 1.12, Fig. 3a). In the remaining four animals, autolysis was already
186 advanced; thus in these fish, presence of parasite DNA could only be shown by real time PCR. No
187 other signs of concurrent disease were detected in these spontaneous deaths, therefore mortality due to

188 PKD was calculated at 15% (13/85). These PKD related mortalities occurred between 45 and 73 days
189 post exposure (p.e., Fig. 2).

190 The surviving 70 brown trout were euthanized after 84 days. Body condition was good (condition
191 factor, mean value: 1.09, SD: 0.11). Thirty-four of the 70 surviving brown trout (49%) showed intact
192 *T. bryosalmonae* by histology or IHC. Infection intensity in these animals varied between mild and
193 moderate, at a mean intensity of 1.5 (SD: 0.65). By real time PCR, *T. bryosalmonae* DNA was
194 detected in additional 12 brown trout.

195 Thirty-seven animals presented different pattern of kidney pathology. Sixteen trout showed acute renal
196 changes with extrasporogonic parasites in the interstitium and intravascular thrombi composed of
197 parasite stages, fibrin and mainly macrophages and few lymphocytes. Ten animals showed chronic
198 active changes like interstitial fibrosis, tubulonephrosis and tubuloneogenesis together with an acute
199 response to the disease as described above. In these animals, parasites were present in the interstitium,
200 in the vessels, but also in the tubular lumen (Fig. 2d). However, detectable numbers of intratubular
201 parasites were low. Six animals showed only solitary parasites in the interstitial tissue with only focal
202 lesions. In five animals, kidney changes were already chronic (Fig. 3b,c) with spores in the tubular
203 lumen (Fig. 3d) or no detectable parasites in vessels or the interstitial tissue. No signs of concomitant
204 disease were present in any of the 70 animals.

205 Overall, PKD incidence was 69% (59 out of 85 exposed brown trout) after three months of exposure
206 (Table 1), which was not significantly different to PKD prevalence in wild brown trout (see below).

207

208 **Field sampling**

209 *Infection prevalence, intensity and renal pathology*

210 In the Ehrenbach, none of the examined brown trout showed any signs of a *T. bryosalmonae* infection,
211 neither by histology, immunohistochemistry nor by real time PCR. Kidney morphology was within
212 normal limits in all examined fish.

213 In the Wutach, at the upstream and downstream location, PKD prevalence was 82% and 80%,
214 respectively (Table 1). Infection intensity varied between mild and severe, with a mean value of 2.8

215 and 3.5, respectively (Table 1). At the upstream location, infection with *T. bryosalmonae* was mostly
216 associated with moderate to severe acute kidney lesions (Fig. 3a, Table 1). The mean pathology score
217 was 3.6 (Table 1). One animal showed acute renal lesions and an interstitial severe fibrosis, interpreted
218 as a chronic active lesion. At the downstream location, all infected animals showed acute kidney
219 lesions. The mean pathology score was 3.75. At the up- and at the downstream location, parasites were
220 located in the interstitial tissue and in the vessels.

221 Chi-square analysis comparing PKD prevalence between the two field sampling groups up- and
222 downstream of the cage and the PKD incidence in the cage showed no significant group differences
223 ($p=0.8921$).

224

225 **DISCUSSION**

226 Previous studies hypothesized that increased PKD related mortality is a major contributor to the
227 decline of brown trout populations (Wahli et al. 2008, Zimmerli et al. 2007, Schmidt-Posthaus et al.
228 2013), and YOY brown trout seemed to be especially affected (Burkhardt-Holm et al 2005, Hari et al.
229 2006). In several brown trout populations in Switzerland, lower YOY brown trout densities could be
230 correlated to the occurrence of PKD (Borsuk et al. 2006). In other countries (e.g. Denmark, Norway),
231 the effect of *T. bryosalmonae* infections on the population level is still under discussion (Skovgaard
232 and Buchmann 2012, Mo et al. 2011). *T. bryosalmonae* positive brown trout were found in high
233 numbers in different fish populations by PCR, however clinical signs were only rare (Skovgaard and
234 Buchmann 2012, Mo et al. 2011). Therefore, the authors discussed that *T. bryosalmonae* infections are
235 not always detrimental for wild fish. However, mortality rates related to particular diseases in wild fish
236 populations are difficult to measure as usually no post-mortem data can be collected. Therefore, the
237 aim of this study was to evaluate PKD related pathology and mortality in YOY brown trout kept in a
238 cage in the river Wutach. Interestingly, mortality stayed as low as 15% in our experiment, despite the
239 fact that 69% of caged fish became infected during the experimental period. In temperature controlled
240 aquaria using rainbow trout, mortality up to 85% was shown at constant water temperatures of 18°C
241 (Schmidt-Posthaus et al 2012) over a time period of 75 days post exposure. After 47 days p.e.,

242 cumulative mortality already reached 77% (Bettge et al. 2009). In our cage experiment, PKD related
243 mortality in brown trout occurred between 45 and 73 days p.e. (Fig. 2). Therefore, onset of mortality
244 was delayed compared to laboratory experiments using rainbow trout (Bettge et al. 2009). Species
245 differences could be an explanation for this difference with brown trout being less sensitive to the
246 infection. Water temperature higher than 15°C is thought to be critical for disease related mortality
247 (Bettge et al. 2009, Okamura et al. 2011). In our experiment, daily mean water temperature exceeded
248 15°C for 39 days inside the cage. Fish were exposed relatively late, starting middle of July, whereas
249 native YOY trout are exposed much earlier to infestation. Low mortality could therefore also be due to
250 insufficient infection rate due to low spore density in the water. Spore release by bryozoan seems to be
251 undulating instead of continuous with a peak value in June / July (Hanna Hartikainen, pers. comm.).
252 Further research is needed to investigate this possible impact, e.g. by exposing brown trout in river
253 water over the whole season with regular samplings during the exposition period to monitor disease
254 development in exposed brown trout. However, incidence of infected animals at the end of our
255 experiment was 69%, similar ($p>0.05$) to the disease prevalence found in wild brown trout at the
256 nearby locations in the Wutach (80 and 82%).
257 Additionally, lesions in caged fish were comparable to those occurring in wild fish in the same river
258 during the time period of the experiment, severity of pathology was advanced in wild fish compared to
259 caged animals. However, whereas wild brown trout and caged brown trout dying during the
260 experiment showed acute kidney changes with intravascular extrasporogonic parasites, as well as
261 parasites in the renal interstitium, caged fish sampled at the end of the experiment showed a high
262 percentage (41%) of chronic or chronic active lesions with spores already present inside renal tubuli.
263 This indicates spore translocation into the tubular lumen (sporogonic stage) and spore excretion. The
264 low number of detectable spores in the tubular lumen by immunohistochemistry can be due to the fact
265 that the used monoclonal antibody (anti-*Tetracapsuloides bryosalmonae* (PKX) antibody, AquaMAB-
266 P01, Aquatic Diagnostics Ltd., Stirling, UK) only partly detects sporogonic stages of the parasite, and
267 therefore the number of intratubular spores was probably underestimated. Between the sampling of
268 wild fish and the end of the cage experiment, there was a time delay of 4 weeks, which might explain

269 this morphological difference with more chronic stages in caged animals. Therefore, these animals
270 seemed to be survivors, already within a recovery stage of the disease. Unlike rainbow trout, brown
271 trout are able to excrete intact spores which can re-infect bryozoa (Morris & Adams 2006, Grabner &
272 El - Matbouli 2008). In the field, additional stressors, like concurrent diseases, can retard parasite
273 development in the kidney and the kidney regeneration process in wild brown trout (Schmidt-Posthaus
274 et al. 2013). Food competition can also be regarded as one additional stressor in the field. This stressor
275 was not present in our study as animals in the cage were fed with artificial food which was also
276 reflected by the good body condition of caged brown trout.
277 Our results suggest that PKD in brown trout does not always result in high mortality under natural
278 conditions. Additional research is needed to confirm these indications and to investigate possible
279 applications on other river systems.

280

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284

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369 **List of figures**

370 Fig. 1: a. Location of field sampling sites (grey bars), cage (black rectangle) and location of
371 temperature logger in the Wutach and the Ehrenbach (grey triangles). Black bars indicate barriers
372 which are not traversable by brown trout. b. Temperature curve in the Ehrenbach. c. Temperature
373 curve in the Wutach in the cage. Thin lines indicate 15°C which was shown to be critical for PKD
374 related clinical signs and mortality in trout. Thick dark line indicates period where temperature stayed
375 above 15°C.

376

377 Fig. 2: Survivorship curve of brown trout (*Salmo trutta fario*) kept in the cage in the river Wutach.

378 P.e.= post exposure in river water

379

380 Fig. 3: Histological lesions found in brown trout kept in the cage in river water, a. acute renal change
381 with thrombus formation (arrow), thrombus consists of mainly macrophages, fewer lymphocytes and
382 parasites (open arrowhead), b. chronic renal changes with fibrosis around tubuli and vessels (open
383 arrowheads), c. higher magnification of chronic changes (star) with fibrosis and infiltration with
384 macrophages and eosinophilic granular cells, HE stain. d. IHC investigation showing positive parasite
385 spores (arrowhead) in the tubular lumen. Bars (a,c) = 25 µm, (b) = 50 µm, (d) = 10 µm

List of tables

Table 1: *Salmo trutta fario*, field sampling and cage experiment, different locations of sampling, length, prevalence of infected brown trout, infection intensity (shown as mean values \pm standard deviation) and associated renal pathology (shown as mean values \pm standard deviation), PKD related mortality, nk = not known. Histopathological changes of the kidney were graded as 0 (no), 1 (scattered), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe) or 6 (severe). Presence of *T. bryosalmonae* was classified as 0 (no parasites present on whole slide), 1 (single parasites), 2 (mild infection rate), 3 (mild to moderate infection rate), 4 (moderate infection rate), 5 (moderate to severe infection rate) or 6 (severe infection rate) at a magnification of 200 to 400x.

Location			<i>T.b. in brown trout</i>					
		n	Length (cm) (Mean \pm SD)	Prevalence (%)	Infection intensity (Mean \pm SD)	Pathology (Mean \pm SD)	Mortality (%)	
Field sampling	Wutach	Upstream location	11	10.2 \pm 1.0	82	2.8 \pm 1.6	3.6 \pm 1.7	nk
		Downstream location	10	10.4 \pm 0.8	80	3.5 \pm 2.1	3.75 \pm 1.7	nk
	Ehrenbach		15	9.3 \pm 1.5	0	0 \pm 0	0 \pm 0	nk
Cage experiment	Fish farm	Starting control	5	8.8 \pm 0.8	0	0 \pm 0	0 \pm 0	0
	Fish farm	Negative control	10	11.7 \pm 1.8	0	0 \pm 0	0 \pm 0	0
	Wutach	Caged animals	85	11.2 \pm 1.3	69	1.6 \pm 1.1	2.3 \pm 1.4	15