

1 **Growth and Welfare of African catfish (*Clarias gariepinus* Burchell,**
2 **1822) under dietary supplementation of the mixed layer clay mineral**
3 **montmorillonite–illite/muscovite (1g557) in commercial aquaculture**

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13
14 **Abstract**

15 Juvenile African catfish (*Clarias gariepinus*) was reared under two commercial recirculation
16 aquaculture conditions (E1, E2). The mixed layer clay mineral montmorillonite–
17 illite/muscovite (1g557) was applied as feed additive over 70 d. Most efficient growth was
18 observed for the 0.5% 1g557 group, performing 0.8% (E1) and 3.2% (E2) better than the
19 catfish basic diet control. In E1, the leptokurtic distribution with a negative skewness also
20 demonstrated the highest number of larger sized fish per batch. Mortality was similarly low in
21 all treatment groups (E1: 3.6 - 4.9%/ E2: 2.5 - 4.8%). In E1, the number of skin lesions
22 decreased considerably after 29 d in the 0.5% and 2.0% groups (from 1.9/1.5 to 0.8/0.8
23 lesions per fish, respectively), while it remained nearly constant in the control (from 1.5 to
24 1.2) ($p < 0.05$ between control and 2.0% group). After 70 d, the number of lesions significantly
25 decreased to 0.4 and 0.5 in the 0.5% and 2.0% groups, with minor changes in the control
26 (0.9 lesions per fish). Independent sampling in E2 verified these findings, with the number of
27 lesions decreasing to 0.3 and 0.6 in the 0.5% group and the control. In E1, cortisol and
28 glucose increased strongly in all groups due to induced stress; this was not evident in E2
29 based on a different sampling procedure. Additional blood parameters were not significant in
30 both experiments, suggesting no negative effects on the African catfish organs and
31 metabolism. Supplementation of 0.5% 1g557 to commercial African catfish diet increases
32 fish growth performance, reduces size variance, and supports fish welfare under different
33 commercial aquaculture conditions.

34
35 **Key words:** African catfish, clay mineral, feed additive, feed supplements, fish welfare,
36 montmorillonite, 1g557.

43 1. Introduction

44 African catfish (*Clarias gariepinus* Burchell, 1822) is a warm water aquaculture fish
45 with increasing commercial importance worldwide. The production of *C. gariepinus* in
46 recirculating aquaculture systems (RAS) has increased also in Germany between 2011
47 (318,575 t year⁻¹) and 2019 (930,246 t year⁻¹) (Destatis, 2012 - 2020). Due to its high
48 tolerance towards adverse water conditions, such as low oxygen (Belão et al., 2011), high
49 ammonium, nitrite, and nitrate concentrations (Schram et al., 2014; Roques et al., 2015;
50 Păpuc et al., 2019), this fish can be reared under high stocking densities, reaching up to 500
51 kg m⁻³ (Van de Nieuwegiessen et al., 2009).

52 Clay minerals have been attributed to positive effects in aquaculture, ranging from
53 enhanced water conditioning, detoxification to increased growth, health or well-being in
54 farmed aquatic animals. The physical and chemical properties of clay minerals are
55 determined by their chemical composition and the spatial crystal structure. Ions in this
56 layered structure can be exchanged or easily hydrated. Thus, clay minerals are able to
57 adsorb different ions, such as nitrogen compounds and phosphates (Eturki et al., 2012), but
58 also fatty acids, nucleic acids, or proteins (Edzwald et al., 1976; Heimann, 2010). According
59 to Attramadal et al. (2012), the addition of clay minerals (mainly illite) led to water quality
60 improvements during the breeding of Atlantic cod larvae (*Gadus morhua*), where dissolved
61 organic material was bound, thereby reducing the bacterial load and, in most cases, larval
62 mortality. Montmorillonite in feed adsorbs mycotoxins (toxic metabolites of fungi) (Desheng et
63 al., 2005; Pasha et al., 2008; Hassan et al., 2010; De Mil et al., 2015) or the herbicide
64 glyphosate (Houry et al., 2010); both posing a growing threat in animal farming, causing
65 neurotoxic or carcinogenic effects, developmental disorders, decreased weight gain, an
66 impaired immune system, or increased mortality (Gill et al., 2018; Marijani et al., 2019;
67 Oliveira & Vasconcelos, 2020; Koletsi et al., 2021). Some mycotoxins might accumulate in
68 tissues and may also reach end consumers (Deng et al., 2010; Anater et al., 2016; Oliveira &
69 Vasconcelos, 2020). Palm et al. (2015, 2021) described an increased survival rate, higher
70 final weights, more efficient feed conversion and reduced size variance of White Leg Shrimp
71 postlarvae (*Litopenaeus vannamei*) under application of feeds containing 2%
72 montmorillonite-illite/muscovite (1g557), or a mixture of 2% of this clay mineral and 2% of the
73 microalgae *Chlorella vulgaris*. Positive influence on growth performance and feed digestibility
74 were also described for Nile tilapia (*Oreochromis niloticus*) fed with supplemented (Cu²⁺-
75 exchanged) montmorillonite (Hu et al., 2007, 2008). Eya et al. (2008) tested feeds
76 supplemented with 0%, 2.5%, 5%, and 10% bentonite in rainbow trout (*Oncorhynchus*
77 *mykiss*). After 90 d, 5 and 10% bentonite supplementation significantly improved growth
78 parameters, such as percent weight gain, specific growth rates (SGR), and feed efficiency.

79 The welfare of African catfish has been assessed by analysing the behaviour,
80 external injuries (skin lesions), cortisol, glucose, lactate, growth and mortality (Martins et al.,
81 2006a, 2006b; Van de Nieuwegiessen et al., 2008, 2009; Baßmann et al., 2017, 2020).
82 Rearing fish under very high stocking densities might affect fish welfare and survival
83 negatively, suggesting application of feed additives to increase fitness and survival in return.
84 Since 2016, montmorillonite-illite has been approved as technological feed additive by the
85 EU-regulation EU 2016/1964 under the abbreviation 1g557 (European Commission, 2016),
86 having positive effects onto survival and growth performance of White Leg shrimps (Palm et
87 al. 2015, 2021). The present study analysed external injuries, growth, mortality, and blood
88 parameters of African catfish under supplementation of 1g557 as a feed additive in order to
89 promote growth and welfare of this species under commercial production conditions in two
90 different aquaculture facilities.

91 2. Material and methods

92 2.1 Production systems and maintenance

93 Two experiments (E1 and E2) were conducted, E1 at the aquaculture research facility
94 'FishGlassHouse' of the University of Rostock, and E2 at a local catfish farmer (Fischzucht
95 Abtshagen, Mecklenburg-Western Pomerania, NE Germany). Both used recirculation
96 aquaculture systems (RAS) for catfish production at a commercial scale.

97 The system used in E1 has been previously described by Palm et al. (2018). It
98 consists of nine identical rearing tanks, each measuring (L x W x H) 1.8 x 1.0 x 0.7 m, 1.26
99 m³. The process water is cleaned through a settling tank (1.3 m³, equipped with lamella
100 inserts, specific surface area of 105.00 m² m⁻³) and a trickling filter (total volume: 5.9 m³,
101 specific surface area: 125 m² m⁻³), subsequently collected in a sump (2.7 m³), before getting
102 returned to the fish tanks. The RAS contained a total of 15.1 m³ water. Regular water
103 exchange was done with tap water (approx. 624 L d⁻¹ = 4.1% of the total volume). The
104 settling tank was cleaned weekly. The temperature was set to 27°C. The pH was adjusted by
105 adding calcium hydroxide as soon as it dropped below 5.5.

106 In E2, six identical rearing tanks (L x W x H: 1.37 x 0.94 x 0.9 m, 1.16 m³) which were
107 part of a larger RAS were used. The RAS was equipped with two settling tanks (each
108 0.95 m³, lamella inserts, specific surface area of 125 m² m⁻³) and two biofilters (one trickling
109 filter, approx. 14.1 m³, specific surface area: 125 m² m⁻³ and one moving bed filter, 5.1 m³,
110 biocarrier volume: approx. 2.75 m³ with biocarrier >750 m²/m³ total surface area). The water
111 was collected in a sump (2.5 m³). This RAS contained a total of 18.8 m³ water. Water
112 exchange was done twice a week when the settling tanks were cleaned (each time 1.9 m³).

113 In both experiments, temperature, oxygen concentration and saturation, pH, electric
114 conductivity (EC), salinity, and redox potential were recorded daily (each in triplicates) with a
115 portable multimeter (Hach-Lange HQ40D, Germany) at the settling tanks influx, its efflux, and
116 behind the trickling filter. Twice a week, water samples were analyzed in triplicates by using
117 an automatic photo-analyzer (Gallery™, Thermo Fisher Scientific) for ammonium/ ammonia
118 (NH₄⁺/ NH₃), nitrite (NO₂⁻), nitrate (NO₃⁻), and ortho-phosphate (PO₄³⁻).

119 120 2.2 Experimental feeds

121 The experimental feeds (feed mill Spezialfuttermittelwerk Beeskow GmbH, Germany)
122 were either mixed with 0.5% or with 2.0% 'Friedland clay' 1g557 as a registered
123 technological feed additive under EFSA (2014). The composition of the basic catfish feed is
124 given in Tab. 1, with the ingredients wheat, fish meal 70M, poultry meal, HP-soya extract
125 grist, haemoglobin powder, hydrolyzed feather meal, pea protein, monocalcium phosphate
126 and additional vitamins A, C, D, E. After mixing the respective amount of 1g557 (Palm et al.
127 2021) to the basic feed, fish oil and water was added according to the manufacturer's
128 recommendation, pelleted (feed pellet laboratory press type 14-175 by Amandus Kahl GmbH
129 & Co., Germany), and deep frozen at -20°C until feeding. Feed processing was repeated five
130 times during the experiments to obtain fresh feeds. The pellet stability in water was tested
131 beforehand and regarded as sufficient.

132 The clay mineral 1g557 originated from the open cast mine near Friedland in
133 Mecklenburg-Western Pomerania, Northern Germany, and is a mixture of different minerals,
134 dominated by 35 - 53% swellable montmorillonite/illite, about 30% non-swellable
135 illite/muscovite, and < 20% koalinite, and quartz. Siderite, pyrite and other minor constituents
136 (< 1%) are also present (Henning & Kasbohm, 1998; EFSA, 2014; FIM Biotech, 2017). The

137 empirical formula is $\text{Na}_{0.03}\text{Ca}_{0.04}\text{K}_{0.16}(\text{Al}_{1.87}\text{Fe}_{0.16}\text{Mg}_{0.16})(\text{Si}_{3.31}\text{Al}_{0.69})\text{O}_{10}(\text{OH})_2$ (EFSA, 2014). By
 138 definition, Friedland clay is not a true bentonite, although its physical properties are
 139 determined by montmorillonite, which is the main component in bentonites. Compared to
 140 other bentonites, Friedland clay has a lower swelling capacity and a lower specific surface
 141 area (Henning & Kasbohm, 1998).

142

143 Table 1: Composition of experimental feeds according to manufacturer's specifications.

Nutrients	Coppens Special Pro EF 4.5	Catfish experimental feed (feed mill Beskow)		
	adaption period	control group	0.5% group	2.0% group
Crude protein [%]	42.0	45.2	44.974	44.296
Crude fat [%]	13.0	15.0	15.0	15.0
Carbohydrates [%]	not specified	19.6	19.502	19.208
Crude ash [%]	7.8	5.1	5.075	4.998
Crude fiber [%]	1.5	1.4	1.393	1.372
Phosphorus [%]	1.14	1.0	0.995	0.980
Digestible energy [MJ kg ⁻¹]	17.1	20.1	20.0	19.7
1g557 [%]	0	0	0.5	2.0

144

145 2.3 Fish stocking

146 In E1, 926 presorted juvenile African catfish were obtained on 1st Feb. 2019 from
 147 Fischzucht Abtshagen and stocked randomly into the nine tanks with 103 fish/tank (one tank
 148 with 102 fish, appr. 2.5 kg/m³). Respectively, three tanks were allocated to one of three
 149 treatment groups: 0.5%, 2.0%, and control group. The fish from the 0.5% and 2.0% groups
 150 had significantly different mean initial weights and body lengths. Also the 2.0% group had a
 151 significantly different condition index to both other groups (Tab. 3).

152 In E2, 618 juvenile African catfish were bred directly at Fischzucht Abtshagen,
 153 presorted and stocked on 24th Apr. 2020 into six tanks (103 fish/ tank). Three tanks each
 154 were allocated to one of two treatment groups, 0.5% and control. These fish had insignificant
 155 mean initial weights, body lengths, and condition indices (Tab. 4).

156 In both experiments a randomized block design in triplicates was used. During an
 157 adaptation period of 31 d, all fish were fed with a regular commercial catfish diet (Coppens
 158 Special Pro EF 3 - 4.5 mm, Tab. 1). On 5th Mar. 2019 (E1) and on 26th May 2020 (E2) this
 159 diet was changed by switching to the pelleted experimental feeds (Tab. 1). The
 160 unsupplemented African catfish feed mixture (feed mill Beskow) was taken as control. In the
 161 supplemented feeds, replacement of 0.5% or 2.0% of the regular feed with 1g557 reduced
 162 the nutrient content by maximum 0.5% or 2.0%, and the digestible energy by 0.1 - 0.4%. The
 163 amount of feed given per day was based on an existing commercial feeding protocol
 164 (between 3.9 and 1.5% of fish body weight depending on the growth stage, Fischzucht
 165 Abtshagen). Feeding took place every two hours between 07:00 p.m. – 05:00 a.m. by using
 166 automatic feeders.

167

168 2.4 Sampling

169 After the adaptation period, samplings were performed every four weeks. In E1, the
 170 first sampling (T0) was conducted on 4th Mar. 2019 by measuring body weights, body
 171 lengths, the initial levels for plasma cortisol, blood glucose, and external injuries (skin lesions
 172 and fin erosions as result of aggressive behavior) as a sub-sample (11 fish per tank = 33 fish
 173 per treatment group). The next sub-sample (15 fish per tank = 45 fish per treatment group)

174 was taken over three days from 1st Apr. – 4th Apr. 2019 (T1), whereby fish growth and welfare
175 parameters were recorded again. A further sub-sample (15 fish per tank = 45 fish per
176 treatment group) followed from 1st May – 3rd May (T2) recording same parameters. The final
177 sampling (T3) was done on 13th May 2019 by taking body weights, body lengths, the number
178 of external injuries and mortality from all remaining fish.

179 In E2, the first sampling (T0) was conducted on 25th May 2020 by measuring the
180 same parameters as in E1, but using three unstressed fish and three stressed fish per tank
181 (18 fish per treatment group). The next sub-sample followed on 22nd Jun. 2020 (T1) with
182 measuring the same parameters in an equal sample size as before. A further sub-sample
183 followed on 22nd July 2020 (T2). The final sampling was done on 3rd Aug. 2020 (T3) by taking
184 weights, lengths, number of external injuries from all remaining fish and the additional blood
185 parameters (see above).

186 Since some fish were removed from the experiment after each sampling, feed
187 conversion ratios (FCR) were calculated for each sampling date ($FCR = TFI/W_t - W_0$ with TFI
188 = total feed intake (SI Einheit), W_0 = initial fish weight (SI Einheit), and W_t = final fish weight
189 (SI Einheit)). All remaining fish in the tanks were considered. The condition index (condition
190 index = fish mass [g] * 100/ fish length [cm]³) was determined at stocking, T0, and T3. At
191 stocking and T3 all fish were sampled (E1: at stocking: 309, 308, 309; T3: 166, 164, 171; E2:
192 at stocking: 309 each; T3: 172, 163); at T0 sub-samples of 33 fish from each group were
193 taken in E1 and E2.

194 2.5 Blood parameters

195 To compare fish welfare, the mortality, growth performance, number of external
196 injuries and the blood parameters plasma cortisol and blood glucose were analyzed. Prior to
197 samplings in E1, the water level of each rearing tank was reduced to approx. 20 cm and all
198 fish from this tank were removed with nets and transferred into 100 L-tubs (confinement
199 stress). This was done in order to prevent continuous catching stress, with potentially
200 different individual time periods inside the fish tanks until blood sampling, affecting the
201 cortisol response. The transfer and maintenance inside the sorting tubs is a stressor
202 simulating regular aquaculture procedures. Afterwards, 15 randomly chosen fish per tank (45
203 per group) were stunned via brain percussion, killed by cutting the gills, and blood sampled
204 over their caudal vessels. Blood glucose was measured *in situ* using test stripes (Accu
205 Check Aviva). Approx. 0.5 mL blood was transferred to reaction tubes with a coated
206 coagulation inhibitor (5.4 mg K-EDTA) and stored on ice. The blood samples were
207 centrifuged (1,250 rpm, at 4°C, for 10 min, Hettich Universal 320 R) and the plasma phase
208 was used for cortisol ELISA (Cusabio, fish cortisol, sensitivity: 0.0023 ng mL⁻¹) according to
209 the manufacturer's instructions. The plasma samples were analyzed by using a micro-plate
210 reader at 450 nm (iMark, Bio-Rad).

211 In E2, nine fish were directly taken from each of the six tanks, stunned, killed, and
212 subsequently blood sampled. This procedure was conducted within 10 min to get a proper
213 indication of the cortisol baseline (reflecting unstressed fish). Cortisol starts to rise within a
214 few minutes after inducing acute stress (Wendelaar Bonga, 1997). Afterwards, all remaining
215 fish were treated as in E1; stress was induced by water level reduction to approx. 20 cm,
216 followed by the catching process and confinement. Then, nine fish per tank were stunned,
217 killed, and blood sampled as described for E1 (reflecting stressed fish, without exactly
218 considering the temporal influence of stress or the stress intensity).

219 Additional blood samples (approx. 3.0 mL in total) were taken to analyze hematocrit,
220 leucocytes, erythrocytes, aspartate aminotransferase (AST), glutamate dehydrogenase

221 (GLDH), cholesterol, triglycerides, urea, sodium, potassium, calcium, chloride, phosphate,
222 glucose, total protein. The concentrations of sodium, potassium and chloride were measured
223 by using an ion-selective electrode. All other chemical blood parameters were quantified by
224 photometry/flow cytometry. In E1 three fish were sampled per group (one per tank) at T0, T1
225 and T3. In E2, two fish per tank (six per group) were sampled at T0, T1 and T3. The sample
226 size differed due to a different amount of blood that could be taken from the fish specimens.

227 The number of skin injuries on body and fins (not on heads due to the stunning
228 method) were recorded always from the same two persons and independently from
229 treatment groups, excluding bias. Only fresh biting wounds penetrating the epidermal layer or
230 reaching down to the underlying tissue were counted. Multiple skin lesions that were
231 obviously related to a single biting attack were counted as one injury, regardless of their
232 individual size. Skin lesions that could not be assigned to a single attack were counted as
233 multiple wounds. Injury marks (scars) were not documented if they were already in the
234 healing process (visible by regenerated epidermal layer or mucus), because they cause no
235 or only minor pressure onto the immune system and hence do not impact fish welfare any
236 more. Sex, weight and length were recorded from the sampled fish. All remaining fish were
237 weighed as a group, counted, and allocated to their respective rearing tanks.

238 2.6 Statistics

239 The resulting data were tested first for distribution. For normal distributed data and
240 three experimental groups, One Way Analyses Of Variance (ANOVA) and post hoc multiple
241 range tests were used; Tukey's-HSD test for variance homogeneity and Dunnett-T3 test for
242 variance inhomogeneity. For not normal distributed data and unequal n, nonparametric
243 Kruskal-Wallis-Test was applied. Parameters of two experimental groups (E2) were analysed
244 by *t*-test if data were normal distributed, otherwise Mann-Whitney test showed significances.
245 All tests were performed with a significance level of $p < 0.05$. In addition, a frequency
246 distribution was performed including range, symmetry, kurtosis, and skewness for fish
247 masses and lengths. These statistical evaluations were conducted by using the SPSS 25
248 (IBM, 2011) statistical software package. Tests performed are specified in the results section
249 of the respective data.

250 251 **3. Results**

252 3.1 Water quality

253 Tab. 2 summarizes the water parameters of E1 and E2. During E1, the water
254 temperature inside the system ranged between 26.6 - 27.8 °C, with highest values at the
255 influxes of the rearing tanks (behind trickling filtration) and lowest at the sedimentation tank
256 influx. The oxygen concentrations were $> 7 \text{ mg L}^{-1}$ ($> 90\%$ saturation) at the inflows of the
257 rearing tanks (after trickling filter) and $> 5.5 \text{ mg L}^{-1}$ (mostly $> 70\%$ saturation) at their effluxes
258 (sedimentation tank influx). During the adaptation phase the pH ranged between 8.3 - 7.1.
259 From T0 - T3, pH-values fluctuated with a general trend towards the low-acid range. The
260 lowest pH of approx. 4 was measured behind trickling filtration. The EC increased slightly,
261 but decreased several times following samplings and resulting water exchanges, before
262 increasing again. EC valued between 855 - 2002 $\mu\text{S cm}^{-1}$. As a result, an almost identical
263 curve for salinity was recognized; the salinity on average was $0.6 \pm 0.1 \text{ ‰}$. The redox
264 potential showed regular fluctuations between 63.3 and 261.2 mV, which reflected the
265 amount of charged molecules absorbed and released into the water. The concentration of

266 NH_4^+ was mostly between 0.1 - 0.2 mg L^{-1} . A peak of NH_4^+ (up to max. 3.7 mg L^{-1}) in the last
 267 third of the experiment was reduced by sampling-related water exchange and the matured
 268 biofilter. NO_2^- was mainly below 0.1 mg L^{-1} . The maximal concentration at the influxes of the
 269 rearing tanks (after trickling filtration) was measured at 0.4 mg L^{-1} . NO_3^- increased steadily
 270 during the experiment from 23.3 up to max. 170.9 mg L^{-1} , but decreased temporary after
 271 water exchange. PO_4^{3-} showed a similar trend as NO_3^- ; however, reaching 8.7 mg L^{-1} at
 272 maximum.

273

274 Table 2: Water parameters (mean \pm standard deviation) in E1 and E2, measured daily (or 2x
 275 weekly*) in the settling tank influx (SI), the settling tank efflux (SE), and the trickling filter
 276 (TF). T = temperature, O_2 = oxygen, EC = electric conductivity, RedOx = redox potential,
 277 $\text{NH}_4\text{-N}$ = ammonium-nitrogen, $\text{NO}_2\text{-N}$ = nitrite-nitrogen, $\text{NO}_3\text{-N}$ = nitrate-nitrogen, $\text{PO}_4\text{-P}$ =
 278 ortho-phosphate.

	E 1			E 2		
	SI	SE	TF	SI	SE	TF
T ($^{\circ}\text{C}$)	27.0 \pm 0.2	27.1 \pm 0.2	27.3 \pm 0.2	28.7 \pm 1.0	28.8 \pm 1.0	28.8 \pm 1.0
O_2 (mg L^{-1})	6.5 \pm 0.5	6.0 \pm 0.7	7.5 \pm 0.2	6.1 \pm 0.7	5.0 \pm 1.0	6.8 \pm 0.5
O_2 (%)	81.7 \pm 5.9	75.3 \pm 9.4	94.2 \pm 2.7	78.8 \pm 8.9	64.6 \pm 11.9	87.7 \pm 5.3
pH	6.6 \pm 1.1	6.7 \pm 1.1	6.8 \pm 1.4	7.0 \pm 0.7	7.0 \pm 0.7	7.1 \pm 0.8
EC ($\mu\text{S cm}^{-1}$)	1254.5 \pm 260.3	1256.5 \pm 259.5	1263.1 \pm 263.1	881.2 \pm 80.5	881.4 \pm 80.8	881.9 \pm 81.0
RedOx (mV)	153.8 \pm 42.6	157.8 \pm 42.0	163.0 \pm 47.4	160.4 \pm 36.7	156.4 \pm 34.0	155.8 \pm 32.6
$\text{NH}_4\text{-N}^*$ (mg L^{-1})	0.6 \pm 0.9	0.6 \pm 1.0	0.5 \pm 0.9	0.7 \pm 2.0	0.7 \pm 2.0	0.7 \pm 1.9
$\text{NO}_2\text{-N}^*$ (mg L^{-1})	0.1 \pm 0.1	0.1 \pm 0.1	0.1 \pm 0.1	0.3 \pm 0.2	0.3 \pm 0.3	0.2 \pm 0.1
$\text{NO}_3\text{-N}^*$ (mg L^{-1})	71.1 \pm 36.4	71.8 \pm 36.1	72.5 \pm 36.1	196.4 \pm 50.3	199.9 \pm 52.9	195.5 \pm 51.5
$\text{PO}_4\text{-P}^*$ (mg L^{-1})	3.8 \pm 1.8	3.9 \pm 1.9	3.8 \pm 1.9	17.2 \pm 9.8	16.6 \pm 9.8	16.6 \pm 9.8

279

280 During E2, the water temperature inside the system ranged between 26.0 and 29.9
 281 $^{\circ}\text{C}$. The oxygen concentration was slightly lower compared with E1, with $> 5.3 \text{ mg L}^{-1}$ ($> 70\%$
 282 saturation) at the inflows of the rearing tanks (after trickling filter) and $> 4.5 \text{ mg L}^{-1}$
 283 (approx. 60% saturation) at their effluxes (sedimentation tank influx). The pH was in a total
 284 range of 8.1 - 4.7. As in E1, EC showed an increasing trend, but did not have a linear
 285 elevation due to water changes. Values between 681 - 1169 $\mu\text{S cm}^{-1}$ were measured. The
 286 mean salinity was $0.4 \pm 0.04\text{‰}$. The redox potential was between 44.8 - 213.9 mV. The
 287 amount of NH_4^+ ranged mostly between 0.002 - 0.7 mg L^{-1} ; a peak up to 9.4 mg L^{-1} occurred
 288 in the last week of E2. NO_2^- showed a higher range compared with E1, but was $< 1.1 \text{ mg L}^{-1}$
 289 after sedimentation (before biofiltration) and $< 0.6 \text{ mg L}^{-1}$ inside the fish tanks (after
 290 biofiltration). Similar to E1, NO_3^- increased steadily, from 106.8 up to max. 284.0 mg L^{-1} , but
 291 decreased temporary after water exchange. PO_4^{3-} showed a similar trend as NO_3^- ; it was
 292 mostly $< 20 \text{ mg L}^{-1}$, with a maximum of 47.1 mg L^{-1} at the end.

293 3.2 Fish growth performance

294 Mean weights, lengths, difference in weight (%), and the condition index of fish at
 295 stocking, T0 and T3 are given for E1 and E2 in Tab. 3. At stocking of E1, fish of the control
 296 group had insignificantly the same size to those of the 0.5% and 2.0% groups. However, due
 297 to the random distribution, fish of the two experimental groups were significantly different
 298 from each other, with fish of the 0.5% group weighing on average 1.3 g more than those of
 299 the 2.0% group.

300 Changing to the test feed at beginning of E1 (T0), sub-samples of 33 fish per group
 301 weighed 107.8 - 112.6 g with lengths of 24.5 - 25.2 cm ($p > 0.05$). After 70 d (T3), the 0.5%
 302 group showed with 484.2 g the highest weight, 0.8% above the control group (480.5 g). The

303 2.0% group was 2.3% below the growth of the control group (469.5 g). Thus, the 0.5% group
 304 tended to show the best average growth performance (insignificant), followed by the control
 305 and the 2.0% group. Both, the fish weight and length were insignificant, with a negative trend
 306 of 2.3% in the 2.0% group.

307 During E2, the fish of control and 0.5% group showed no significant difference in their
 308 size at stocking. At T0, sub-samples of 33 fish weighed 115.2 - 115.6 g with lengths of 25.3
 309 cm ($p > 0.05$). After 70 d (T3), the 0.5% group tended to show again the highest weight
 310 compared with the control group (422.2 g vs. 409.2 g; $p > 0.05$). In E2, the 0.5% group was
 311 3.2% above the weight of the control group (insignificant).

312

313 Table 3: Growth performance (mean, \pm SD) of African catfish in the treatment groups of E1
 314 and E2 fed by different montmorillonite levels (control: C, 0.5%, 2.0%) with Δ [%] as the
 315 difference in weight relative to control (C) and condition index (CI). At T0, a sub-sample of 33
 316 fish was weighed and measured in length; at T3 all remaining fish were weighed and
 317 measured in length; at T0: n.g. = not given since sub-sample; $p \leq 0.05$.

	n	group	weight [g]	length [cm]	Δ [%]	CI [g/cm ³]			
Experiment 1 (E1)									
before stocking and adaptation phase	309	C	30.7 ^{ab}	± 5.8	16.7 ^{ab}	± 1.1	0	0.652 ^a	0.1
	308	0.5%	31.6 ^a	± 6.0	16.9 ^a	± 1.1	+2.9	0.652 ^a	0.0
	309	2.0%	30.3 ^b	± 6.1	16.6 ^b	± 1.1	-1.3	0.661 ^b	0.0
T0 (start of experiment)	33	C	107.8 ^a	± 17.2	24.8 ^a	± 1.5	n.g.	0.7 ^a	0.1
	33	0.5%	112.6 ^a	± 16.2	25.2 ^a	± 1.3	n.g.	0.7 ^a	0.0
	33	2.0%	107.9 ^a	± 17.1	24.5 ^a	± 1.4	n.g.	0.7 ^a	0.0
T3 (after 70 d)	166	C	480.5 ^a	± 89.2	39.2 ^a	± 3.0	0	0.8 ^a	0.1
	164	0.5%	484.2 ^a	± 86.7	39.4 ^a	± 2.8	+0.8	0.8 ^a	0.1
	171	2.0%	469.5 ^a	± 93.3	39.0 ^a	± 2.9	-2.3	0.8 ^a	0.1
Experiment 2 (E2)									
before stocking and adaptation phase	309	C	29.8 ^a	± 3.6	17.0 ^a	± 0.8	0	0.6 ^a	0.1
	309	0.5%	29.7 ^a	± 3.3	17.0 ^a	± 0.8	-0.3	0.6 ^a	0.0
T0 (start of experiment)	33	C	115.6 ^a	± 20.8	25.3 ^a	± 1.6	n.g.	0.7 ^a	0.0
	33	0.5%	115.2 ^a	± 16.4	25.3 ^a	± 1.4	n.g.	0.7 ^a	0.1
T3 (after 70 d)	172	C	409.2 ^a	± 73.1	37.5 ^a	± 2.4	0	0.8 ^a	0.2
	163	0.5%	422.2 ^a	± 76.1	37.9 ^a	± 2.5	+3.2	0.8 ^a	0.2

318

319 In E1, the FCR ranged from 0.66 to 0.97, whereas in E2 it ranged from 0.76 to 1.71
 320 (Tab. 4). A leptokurtic distribution (above 3) with a skewness below 0 represents best batch
 321 growth, revealing highest number of larger sized fish per batch. Highest kurtosis (13.8 fish
 322 length, 5.5 weight) with a skewness of -2.4 (length) and -1.2 (width) was observed in the
 323 0.5% group at the end of E1 after 103 days, with similar length (7.6, -1.6; 7.4, -1.1) and
 324 weight (2.2, -0.8; 3.2, -1.2) distributions for the 2.0% group and the control. At the end of E2,
 325 the batches of the 0.5% group and the control grew similarly slightly platykurtic, with a
 326 kurtosis and a skewness around 0 (length 0.0, -0.1; 0.5, -0.6 and weight -0.2, 0.4; 0.8, -0.1).

327

328 Table 4: Feed conversion ratio (FCR) in the treatment groups of E1 (on top) and E2 (below).
 329 Since a few fish were removed from the experiment in each sampling, FCR cannot be
 330 indicated from initial stocking to T3. FCR is therefore given for each sampling date based on
 331 the respective previous sampling weights.

	group	Stocking - T0	T0 - T1	T1 - T2	T2 - T3
E1	C	0.66	0.78	0.86	0.96
	0.5%	0.66	0.76	0.89	0.97
	2.0%	0.65	0.77	0.91	0.93
	C	0.76	0.95	1.06	1.71

E2	0.5%	0.76	0.94	1.04	1.44
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332

333 3.3 Fish welfare

334 The mortalities in both experiments are given in Tab. 5. During E1, the mortality inside
 335 the control, 0.5%, and 2.0% groups amounted 11, 15, and 9 fish. This result is a percentage
 336 mortality of 3.6%, 4.9%, and 2.9%, respectively (Ø 3.8). During E2, the mortality inside the
 337 control and 0.5% groups amounted 8 and 15 fish, resulting in a percentage mortality of 2.5%
 338 and 4.8%, respectively (Ø 3.7).

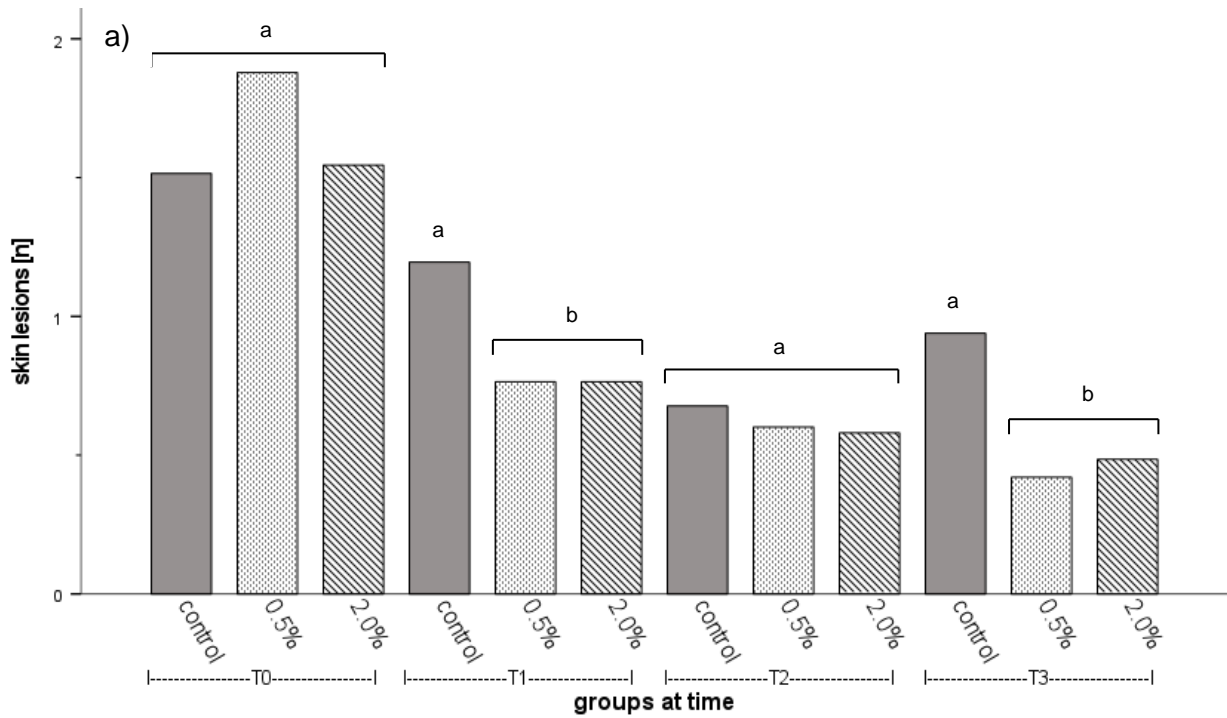
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340 Table 5: Mortality within the treatment groups of E1 and E2, both at T3.

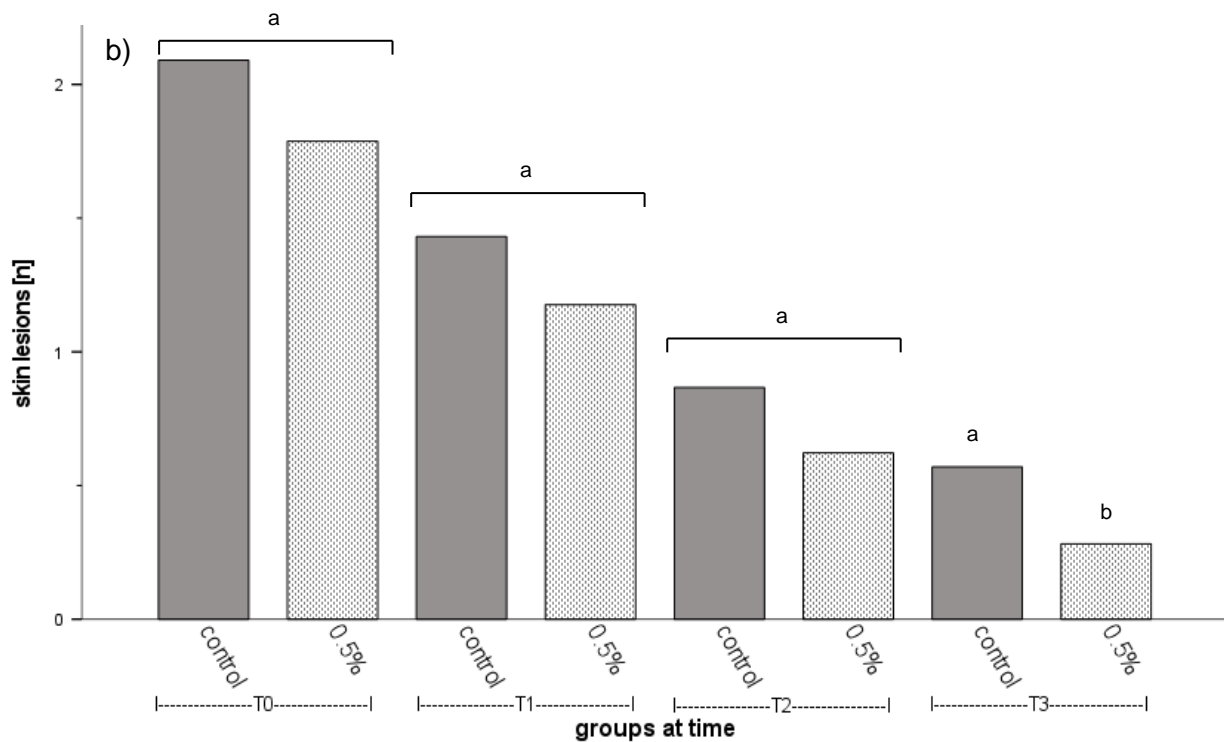
	Groups at T3		
	Control (E1/E2)	0.5% (E1/E2)	2.0% (E1)
Mortality [n]	11/8	15/15	9
Mortality [%]	3.6/2.5	4.9/4.8	2.9

341

342 The number of external injuries decreased in all treatment groups over the run of both
 343 experiments. In E1, however, there was a significant decrease in the number of external
 344 injuries in both experimental groups compared with the control (Fig. 1 a). At T0, the 0.5%
 345 group had a relatively high mean value of 1.9 (\pm 1.8) lesions per individual compared with
 346 both other groups (control group: 1.5 \pm 1.4; 2.0% group: 1.5 \pm 1.5). Due to high standard
 347 deviations, there was no significant difference between the groups. At T1, the number of
 348 lesions in the 0.5% and 2.0% groups decreased to 0.8 (\pm 1.3) and 0.8 (\pm 1.5), respectively, a
 349 significant reduction compared with the control (1.2 (\pm 1.3) ($p < 0.05$) lesions per fish). At T2,
 350 the number of lesions continued to decrease in all groups, insignificant between each other.
 351 At T3, the numbers of lesions in the 0.5% and 2.0% groups decreased to 0.4 \pm 0.8 and 0.5 \pm
 352 0.8, significantly lower compared with the control group (0.9 \pm 1.0). A similar pattern was
 353 observed in E2 (Fig. 1 b). The numbers of external injuries of the control and 0.5% groups
 354 were insignificant at T0, with 2.1 (\pm 1.5) and 1.8 (\pm 1.8) lesions per fish. At T1 and T2, the
 355 groups remained insignificant, with the 0.5% group tending to have fewer lesions than the
 356 control. At T3, the number of lesions per fish was significantly different between the control,
 357 0.6 (\pm 1.2), and the 0.5% group, 0.3 (\pm 0.8).



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Figure 1 a, b: Average number of external injuries of a) three treatment groups (control, 0.5% and 2.0%) in E1, and b) two treatment groups (control and 0.5%) in E2, each at T0 (baseline prior to feed supplementation), at T1 (after 29 d of feed supplementation), at T2 (after 58 d), and at T3 (after 70 d), respectively. Significance $p \leq 0.05$ (Kruskal-Wallis-ANOVA (a); Mann-Whitney test (b)).

In E1, the mean plasma cortisol prior to feed supplementation (at T0) in the control, 0.5%, and 2.0% groups were $22.7 (\pm 14.4) \text{ ng mL}^{-1}$, $28.8 (\pm 15.2) \text{ ng mL}^{-1}$, and $24.7 (\pm 13.1) \text{ ng mL}^{-1}$ ($p > 0.05$). After the diet change (at T1), plasma cortisol in the 0.5% and 2.0% group

369 increased by approx. 249% or 190% (to $100.5 \pm 65.5 \text{ ng mL}^{-1}$ and $71.5 \pm 68.4 \text{ ng mL}^{-1}$,
370 respectively). However, an increase of approx. 344% was also noted in the control group (to
371 $100.8 \pm 68.1 \text{ ng mL}^{-1}$). Significant differences were recorded between the 2.0% group and
372 both others. At T2, plasma cortisol concentrations in the control, 0.5%, and 2.0% groups
373 were at $82.7 \pm 43.6 \text{ ng mL}^{-1}$, $140.8 \pm 53.2 \text{ ng mL}^{-1}$, and $99.2 \pm 76.7 \text{ ng mL}^{-1}$ and so approx.
374 264%, 389% and 302% higher compared with their respective baseline values. Here, the
375 0.5% group was significantly higher than both other groups (Fig. 2 a). In E2, the mean
376 plasma cortisol baselines (at T0) in the control and 0.5% groups were $19.2 (\pm 9.0) \text{ ng mL}^{-1}$
377 and $20.8 (\pm 7.2) \text{ ng mL}^{-1}$ in unstressed fish, while they were slightly increased in stressed fish
378 ($33.3 \pm 17.8 \text{ ng mL}^{-1}$ and $32.3 \pm 15.3 \text{ ng mL}^{-1}$). After the diet change (at T1), mean levels
379 remained nearly identical with $19.7 (\pm 9.4) \text{ ng mL}^{-1}$ and $19.9 (\pm 3.8) \text{ ng mL}^{-1}$ in unstressed
380 fish, and with $25.2 (\pm 8.5) \text{ ng mL}^{-1}$ and $24.4 (\pm 8.1) \text{ ng mL}^{-1}$ in stressed fish. At T2, a minor
381 elevation occurred in all treatment groups. Unstressed fish in the control and 0.5% group
382 showed $32.7 (\pm 13.5) \text{ ng mL}^{-1}$ and $32.3 (\pm 15.3) \text{ ng mL}^{-1}$, while stressed fish showed $29.5 (\pm$
383 $12.1) \text{ ng mL}^{-1}$ and $33.2 (\pm 11.8) \text{ ng mL}^{-1}$ (Fig. 2 b).

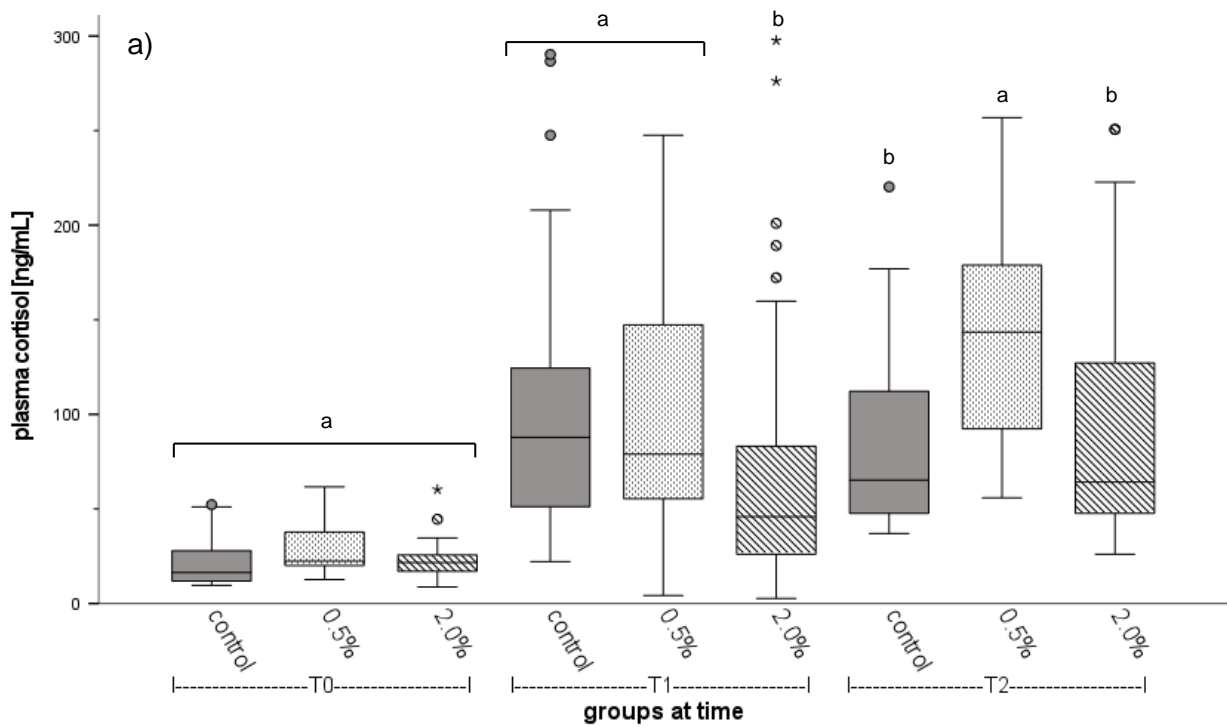
384 In E1, the mean glucose baseline levels were at $2.4 (\pm 0.4) \text{ mmol L}^{-1}$ in the control
385 group and at $2.5 (\pm 0.4) \text{ mmol L}^{-1}$ in both the 0.5% and 2.0% groups, with no significant
386 difference. After switching to the supplemented diets (at T1), the mean glucose of the control
387 group increased to $3.8 (\pm 0.9) \text{ mmol L}^{-1}$, the 0.5% group to $3.6 (\pm 0.8) \text{ mmol L}^{-1}$, and the 2.0%
388 group to $3.4 (\pm 0.7) \text{ mmol L}^{-1}$, with the 2.0% group being significantly lower than the control
389 group. At T2, the glucose of the control group averaged $4.0 (\pm 1.0) \text{ mmol L}^{-1}$, the 0.5% group
390 $4.4 (\pm 1.0) \text{ mmol L}^{-1}$, and the 2.0% group $4.3 (\pm 0.9) \text{ mmol L}^{-1}$, with no significant differences
391 between the groups (Fig. 3 a). In E2, the mean glucose baseline levels (at T0) in the control
392 and 0.5% groups were $2.7 (\pm 0.4) \text{ mmol L}^{-1}$ and $2.8 (\pm 0.6) \text{ mmol L}^{-1}$ in unstressed fish, while
393 they were elevated to $4.0 (\pm 0.5) \text{ mmol L}^{-1}$ and $4.2 (\pm 0.8) \text{ mmol L}^{-1}$ in stressed fish,
394 respectively. At T1, glucose decreased slightly throughout all groups, with mean levels of 2.0
395 $(\pm 0.2) \text{ mmol L}^{-1}$ and $2.1 (\pm 0.3) \text{ mmol L}^{-1}$ in unstressed fish of the control and 0.5% groups,
396 and $3.5 (\pm 0.4) \text{ mmol L}^{-1}$ and $3.9 (\pm 0.7) \text{ mmol L}^{-1}$ in stressed fish, respectively. At T2,
397 glucose of the control and 0.5% groups averaged $2.4 (\pm 0.5) \text{ mmol L}^{-1}$ and $2.5 (\pm 0.5) \text{ mmol}$
398 L^{-1} in unstressed fish, while it was regularly increased to $3.5 (\pm 0.7) \text{ mmol L}^{-1}$ and $3.6 (\pm 0.7)$
399 mmol L^{-1} in stressed fish, respectively (Fig. 3 b). There were no significant differences
400 between the groups within the different sampling events.

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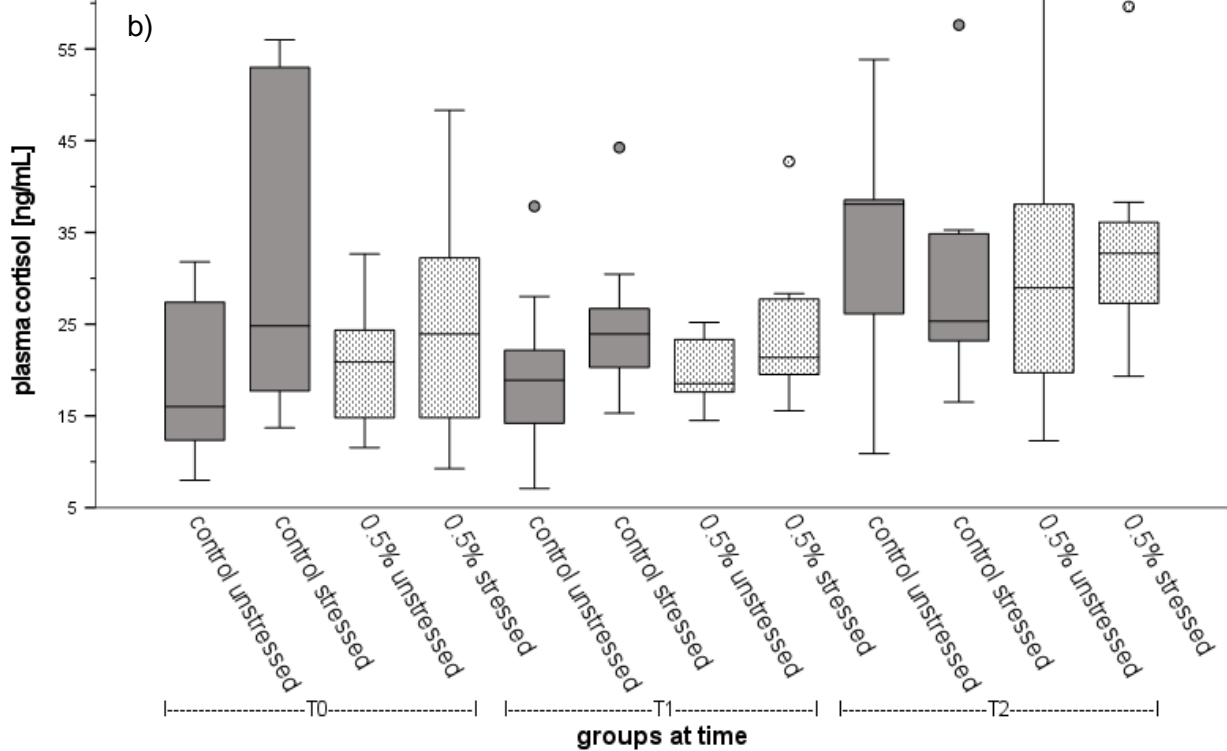
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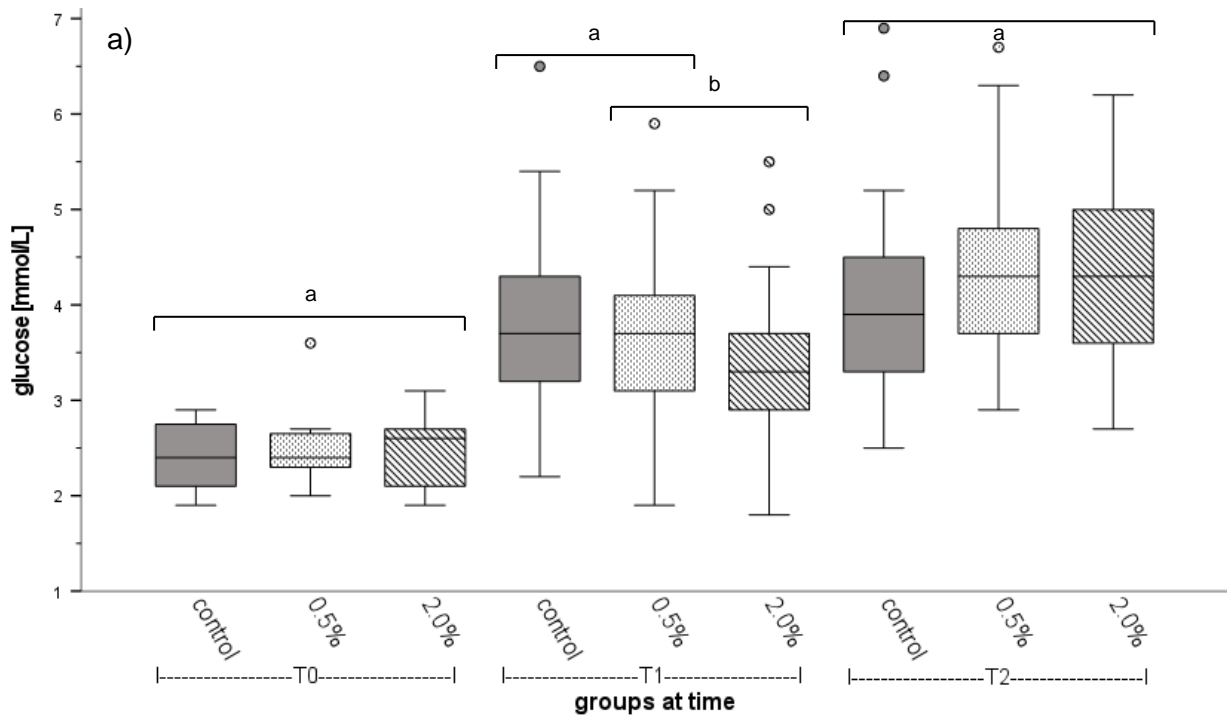
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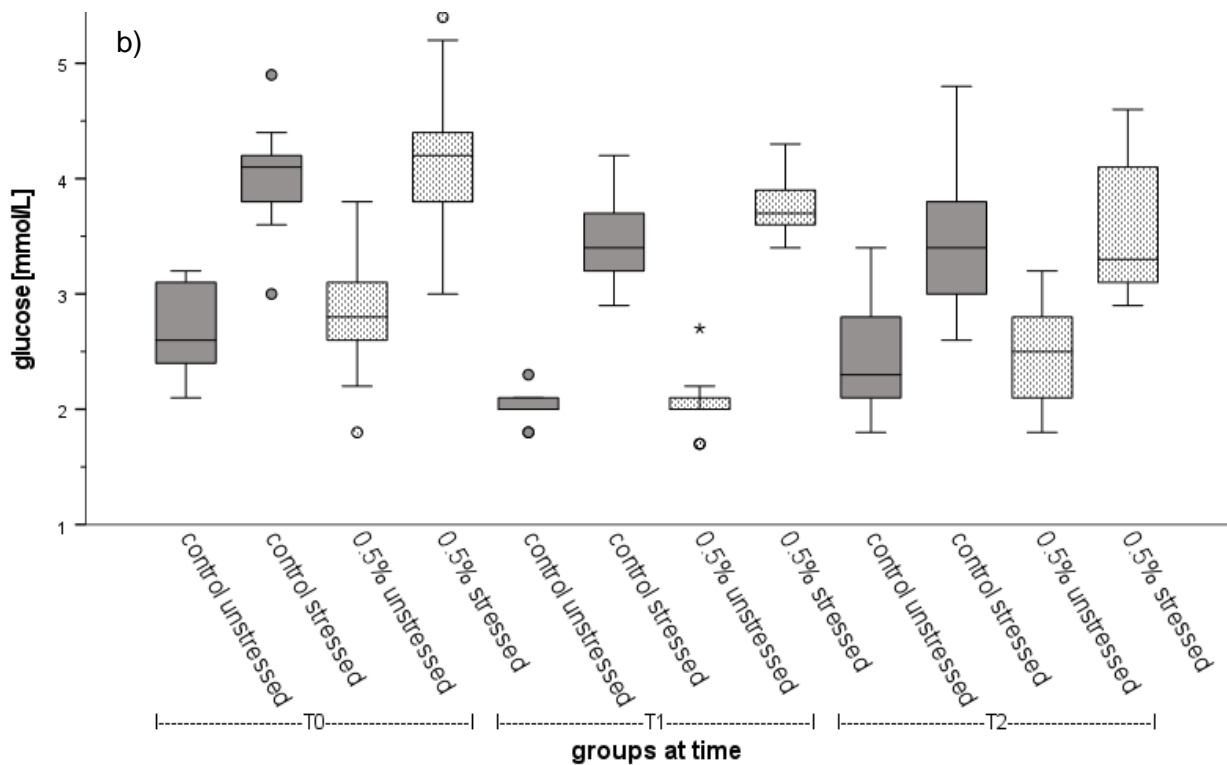
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Figure 2 a, b: Plasma cortisol concentrations a) in three treatment groups (control, 0.5% and 2.0%) in E1 after stress, and b) of unstressed and stressed fish in two treatment groups (control and 0.5%) in E2, each at T0 (baseline prior to feed supplementation), at T1 (after 29 d of feed supplementation), and at T2 (after 58 d), respectively. Asterisk (*) = extreme values; circlets (°) = outliers. In a), at T2, an extreme value of 387.7 ng mL⁻¹ in the 2% group is not illustrated. Significance $p \leq 0.05$ (Kruskal-Wallis-ANOVA (a)). In b) all data were insignificant (t -test or Whitney-Mann test).



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Figure 3 a, b: Blood glucose levels of a) three treatment groups (control, 0.5% and 2.0%) in E1 after stress, and b) of unstressed and stressed fish in two treatment groups (control and 0.5%) in E2, each at T0 (baseline prior to feed supplementation), at T1 (after 29 d of feed supplementation), and at T2 (after 58 d), respectively. Asterisk (*) = extreme values; circlets (°) = outliers. In a), at T2, an extreme value of 8.3 mmol L⁻¹ in the 0.5% group is not illustrated. In b), at T1, an extreme value of 5.6 mmol L⁻¹ in the stressed 0.5% group is not illustrated. Significance $p \leq 0.05$ (ANOVA, Tukey-HSD, Kruskal-Wallis-ANOVA, (a)). In b) all data were insignificant (t -test or Whitney-Mann test).

427 The blood parameters (Tab. 6) aspartate transaminase (AST)/glutamic oxaloacetic
 428 transaminase (GOT), glutamate dehydrogenase (GLDH), total protein, urea, calcium, and
 429 phosphate were not significant in both experiments. The cellular blood components
 430 (leucocytes, erythrocytes, haematocrit) and the chemical blood parameters (cholesterol,
 431 triglycerides, sodium, potassium) (Tab. 7) were also not significant in both experiments.

432

433 Table 6: Liver and kidney blood parameters at T0 (baseline) and T3 (end of the experiment)
 434 in E1 and E2.

	n	group	AST (GOT) [U/l]	GLDH [U/l]	urea [mmol/l]	calcium [mmol/l]	phosphat [mmol/l]	total protein [g/l]
Experiment 1 (E1)								
T0 (start of experiment)	9	base line	333.7 ± 79.7	29.4 ± 3.7	1.1 ± 0.1	2.8 ± 0.1	3.9 ± 0.2	29.9 ± 1.4
T3 (after 70 d)	3	C	208.7 ± 38.2	28.2 ± 3.7	1.1 ± 0.1	3.3 ± 0.2	3.7 ± 0.6	39.3 ± 1.2
	3	0.5%	167.3 ± 12.2	28.1 ± 4.7	1.0 ± 0.1	3.3 ± 0.3	3.3 ± 0.1	38.0 ± 4.1
	3	2.0%	179.0 ± 5.0	22.7 ± 2.1	1.0 ± 0.1	3.2 ± 0.4	3.2 ± 0.4	37.0 ± 2.2
Experiment 2 (E2)								
T0 (start of experiment)	12	base line	150.8 ± 28.7	14.7 ± 2.5	1.3 ± 0.2	3.0 ± 0.1	3.0 ± 0.2	33.7 ± 1.2
T3 (after 70 d)	6	C	109.0 ± 18.8	18.4 ± 2.2	1.1 ± 0.1	3.4 ± 0.1	2.5 ± 0.2	39.5 ± 2.4
	6	0.5%	142.7 ± 48.8	26.5 ± 9.2	1.3 ± 0.1	3.5 ± 0.2	2.8 ± 0.2	41.1 ± 2.2

435

436 Table 7 Cellular blood components and chemical blood parameters at T0 (baseline) and T3
 437 (end of the experiment) in E1 and E2.

	group	leucocytes [G/l]	erythrocytes [T/l]	hematocrit [%]	cholesterol [mmol/l]	triglycerides [mmol/l]	sodium [mmol/l]	potassium [mmol/l]	chloride [mmol/l]
Experiment 1 (E1)									
T0	base line	3.3 ± 2.7	1.1 ± 0.6	21.5 ± 7.3	2.9 ± 0.2	1.8 ± 0.1	124.6 ± 2.6	13.0 ± 1.7	112.0 ± 2.0
T3	C	0.9 ± 0.3	2.0 ± 0.5	26.2 ± 1.3	3.5 ± 0.1	2.0 ± 0.1	128.3 ± 1.2	9.3 ± 1.6	107.0 ± 1.4
	0.5%	1.0 ± 0.2	1.7 ± 0.3	25.8 ± 0.2	3.4 ± 0.3	2.0 ± 0.4	129.7 ± 0.9	8.4 ± 0.5	107.7 ± 0.5
	2.0%	1.0 ± 0.0	1.3 ± 0.4	24.5 ± 0.7	3.1 ± 0.3	1.7 ± 0.2	128.7 ± 1.2	9.5 ± 2.3	110.7 ± 2.1
Experiment 2 (E2)									
T0	base line	3.7 ± 0.8	1.9 ± 0.4	26.7 ± 2.0	2.8 ± 0.3	1.8 ± 0.1	130.0 ± 1.9	7.6 ± 1.3	111.5 ± 1.5
T3	C	13.0 ± 7.5	2.1 ± 0.2	36.3 ± 3.0	3.4 ± 0.2	2.0 ± 0.1	133.5 ± 1.7	7.3 ± 0.6	110.5 ± 1.6
	0.5 %	7.0 ± 3.3	1.7 ± 0.6	34.7 ± 7.1	3.4 ± 0.4	2.0 ± 0.1	133.7 ± 2.0	7.4 ± 0.3	111.0 ± 1.6

438

439 4. Discussion

440 4.1. Culture conditions

441 The water parameters (Tab. 2) provide basic information of the rearing conditions for
 442 African catfish, having also a determining importance on growth and welfare. Despite the
 443 high tolerance of African catfish (Păpuc et al., 2019), it can be assumed that suboptimal or
 444 poor water quality might potentially affect the welfare of this species. Therefore, it was crucial
 445 to provide optimal culture conditions for both experimental approaches inside the two
 446 commercial aquaculture farms.

447 The water temperature in E1 was close to 27°C. There was max. 1.2 °C variation in
 448 the entire system; the temperature variations at the individual sample sites were even closer
 449 and deviated by only 0.6 °C. In E2, the mean temperature was slightly higher with 28.8 °C;
 450 however, both temperature ranges correspond to the optimum for African catfish (Păpuc et
 451 al., 2019). In E1, dissolved oxygen was >90% saturation or >7 mg L⁻¹ (after trickling filtration).
 452 In E2, this was slightly lower with (>70% saturation or >5.3 mg L⁻¹. Masser et al. (1999)

453 recommended maintaining dissolved oxygen levels >60% saturation or >5 mg/L for optimal
454 growth of most warm water species. Thus, the oxygen conditions were in fact optimal during
455 both experiments. A consistently high redox potential was found, also indicating a good
456 oxygen supply.

457 Due to fish respiration, increasing feeding amounts, and bacterial nitrogen
458 metabolism, the pH value usually decreases continuously towards acid conditions (Masser et
459 al., 1999). In E1, the pH started at approx. 8, and dropped temporarily to below 5.5 several
460 times from the fifth week onwards. However, water changes during samplings and regular
461 liming kept the pH mostly above 6.5. A very similar trend was observed in E2. Ndubuisi et al.
462 (2015) reported adequate pH ranges between 5 and 9 for growth and survival of *C.*
463 *gariiepinus*. So, the pH in our study was still in an adequate range. NH_4^+ between 0.1 - 0.2
464 mg L^{-1} as mainly measured in our study can be tolerated very well by African catfish (Păpuc
465 et al., 2019). Under the given temperature and pH ranges only very minor concentrations of
466 toxic unionized ammonia (NH_3) were present (Losordo et al., 1998; Masser et al., 1999). We
467 calculated the unionized form of NH_3 with a maximum of <0.01 – <0.02 mg L^{-1} at pH 8.2 and
468 27°C, being distinctly lower with decreasing pH.

469 Fluctuations of NO_2^- up to 0.4 mg L^{-1} in E1 (0.6 mg L^{-1} in E2) were below or at the
470 recommended maximum (up to 0.6 mg L^{-1}) for African catfish aquaculture, and did not affect
471 growth, well-being, or health (Roques et al., 2015). In E1, NO_3^- (after trickling filter) was on
472 average 72.5 (\pm 36.1) mg L^{-1} , but tended to increase over the experiment to a maximum of
473 170.9 mg L^{-1} . In E2, the mean NO_3^- (after trickling filter) was higher with 190.8 (\pm 56.9) mg L^{-1}
474 and a maximum of 284.0 mg L^{-1} . Schram et al. (2014) recommended not exceeding NO_3^-
475 levels of 140 mg L^{-1} . In E1, nitrate was mostly below, but it exceeded this threshold for about
476 five days and reached 170.9 mg L^{-1} at maximum, which was still considered to be fairly
477 harmless. During E2, the threshold of 140 mg L^{-1} was exceeded for long periods; however,
478 NO_3^- was still relatively low compared to other studies that addressed African catfish in
479 recirculation aquaculture systems. Palm et al. (2018) reported NO_3^- values of 185.5 mg L^{-1}
480 and 125.6 mg L^{-1} at survival rates of 81.4 % and 88.6 % during an entire production periode.
481 Dai et al. (2012) suggested NO_3^- values below 1000 mg L^{-1} and 100% daily water exchange
482 as safe under African catfish production conditions. The survival rates reached 95.1 % -
483 97.5% in the present study. From this perspective, even the slightly elevated NO_3^- levels
484 during E2 might have caused no major effect on the observed welfare status or growth of the
485 African catfish in the present experiments.

486 In summary, water quality in both experiments and aquaculture systems was in a
487 similar range and represented appropriate to optimal culture conditions for the African
488 catfish. Because the physicochemical water parameters were within a similar range as
489 reported by Palm et al. (2018) and the survival rates were high, we conclude that the use of
490 1g557 inside the test feeds did not negatively affected the functionality of both RAS systems.

491 4.2 Fish growth

492 At stocking in E1, the fish mean weights in the control, 0.5% and 2.0% groups were
493 30.7, 31.6, and 30.3 g, respectively ($p < 0.05$ between 0.5% and 2.0% group). At T0, the
494 0.5% group still showed the highest mean weight with 114.0 g, but was insignificant to both
495 other groups. After 70 days of feeding the supplemented diets (T3), the groups were still
496 insignificant with mean weights of 480.5, 484.2, and 469.5 g, respectively. However, a trend
497 was seen that a 2% lower nutrient content in the 2% group resulted in slightly less weight
498 (2.3%) compared to the control group. So the fact that 2% of the feed was replaced by 2%
499 1g557 seemed to have a negative effect on fish growth.

500 A different picture was seen in comparison of the 0.5% group with the control. In E1,
501 there was a slight increase in weight gain of 0.8%, and this was despite the fact that 0.5% of
502 the feed was replaced by 0.5% 1g557. This trend could be verified in E2, where the 0.5%
503 group had an even 3.2% higher weight than the control group ($p > 0.05$). In addition, a lower
504 size variance of African catfish was observed in E1. This is consistent with earlier studies
505 where the addition of montmorillonite or 1g557 achieved good growth performance and lower
506 divergence, even when other species were involved (Hu et al., 2007; 2008; Palm et al.,
507 2015). This suggests that African catfish as well as white-leg shrimps (Palm et al., 2015) can
508 grow more homogeneously under the effect of 1g557 as a feed additive.

509 The Feed conversion ratio during E1 ranged from 0.66 before and 0.76-0.97 after
510 application of 1g557, constantly increasing with increasing fish size from 30-31 (2.5 kg/m^3)
511 until 469-484 g (21 kg/m^3). Palm et al. (2018) reported an FCR of African catfish inside the
512 same aquaculture system between 0.89 and 1.01 in 5 different production phases, from an
513 initial weight of 40-275 g ($2.8\text{-}19.3 \text{ kg/m}^3$) until the final weight of 1496-1780g ($95.5 - 112$
514 kg/m^3). Consequently, the growth performance was as expected during E1, with a slightly
515 better FCR because of smaller sized fish and more extensive production conditions (see
516 Palm et al. 2018) during the entire experiment. During E2, the FCR was higher and not
517 directly comparable because of using a different aquaculture system and cultivation
518 conditions. However, the FCR was already higher during the adaptation phase, indicating
519 that this difference was not caused by the application of the feed additive but originated from
520 the different cultivation system.

521

522 4.3 Fish welfare

523 In the present study 2.9 - 4.9% of the fish did not survive in the experimental groups
524 fed with 0.5% or 2.0% 1g557. Partially, mortalities were slightly lower in the control groups,
525 but in total in a very similar range. So, no negative influence of feed supplementation with
526 1g557 could be detected between the treatment groups. Other studies using regular fish
527 feeds showed similar mortalities, such as 2.5% (van de Nieuwegiessen et al., 2008) or 6%
528 (Baßmann et al., 2020). Palm et al. (2018) reported survival rates up to 90.2% under
529 stocking densities of 199.2 kg m^{-3} , and increasing survival rates under more extensive
530 conditions. This may be the best comparison, as the same RAS was used here with very
531 similar stocking density, same system maintenance, but under common commercial feeds.
532 So, the mortality in our present study can be considered low.

533 An initial increase in agonistic behavior and subsequently a higher number of skin
534 lesions after stocking juvenile African catfish can be considered normal. From our
535 experience, after a few weeks, this usually decreases. Other studies reported 1 - 8 skin
536 lesions per fish (van de Nieuwegiessen et al., 2008; 2009; Manuel et al., 2014). In a three-
537 weeks experiment African catfish fingerlings whose feed contained montmorillonite improved
538 their skin quality, and no adverse effect on growth was determined (Ismaila et al., 2011). In
539 both presented experiments, the highest injury values were recorded at T0. At T1, there was
540 a reduction in the number of external injuries, whereby at E1, the two experimental groups
541 fed with 1g557 showed a significantly decreased number of external injuries than the control.
542 In E2, a similar trend was observed. Finally, significant different numbers of external injuries
543 were recorded independently in both experiments at T3, with all groups fed diets
544 supplemented with 1g577 having approx. half as many injuries as the control groups.

545 Cortisol responses were generally highly diverse, particularly in E1. The mean values
546 ranged between $20 - 140 \text{ ng mL}^{-1}$. Therefore, fewer fish were used for cortisol analyses in
547 E2. In addition, unstressed fish (baseline) and fish after stress induction were used. The

548 plasma cortisol concentrations of fish in E2 were comparable to those at T0 in E1, but apart
549 from that lower. The plasma cortisol levels of stressed fish in E2 were mostly elevated to
550 those of unstressed fish. Solely at T2 the cortisol response of the stressed control was below
551 that of the unstressed control, which cannot be explained, as it was probably not cortisol
552 suppression. The data were also widely scattered in the unstressed 0.5% group, whereas
553 they were closer together in stressed fish. For comparison, Martins et al. (2006b) reported
554 most cortisol levels for unstressed as well as stressed African catfish to be between approx.
555 20 and 100 ng mL⁻¹, with cortisol of unstressed being significantly or at least trending lower
556 than that of stressed fish. Largely, this is consistent with our data. It is likely that the sampling
557 method had a strong influence in our experiments, as all fish were caught and removed from
558 the tanks. Thus, temporal differences as well as a changing stressor intensity on individual
559 fish could easily occur and may have led to different cortisol responses. Glucose ranged from
560 2 to 5 mmol L⁻¹ overall in both experiments, with stressed fish tending to have higher values
561 than unstressed fish in E2. This is also consistent with the results of Martins et al. (2006b).
562 However, we found no major differences between our experimental groups despite
563 significant differences in E1 (at T1), indicating that 1g557 had no adverse effect.

564 The additional blood parameters aspartate transaminase (AST)/glutamic oxaloacetic
565 transaminase (GOT), glutamate dehydrogenase (GLDH), total protein, urea, calcium, and
566 phosphate were not significant in both experiments and indicated regular liver and kidney
567 function. Similarly, the cellular blood components (leucocytes, erythrocytes, haematocrit) and
568 the chemical blood parameters (cholesterol, triglycerides, sodium, potassium) were not
569 significant, suggesting that there were no negative effects of the tested feed additive on the
570 African catfish organs and metabolism.

571

572 **5. Conclusions**

573 The application of the mixed layer clay mineral montmorillonite–illite/muscovite (1g557) in
574 RAS for African catfish has the potential to improve both growth and welfare of the fish
575 without negatively affecting their blood parameters and stress responses or the RAS itself.
576 After 70 days of cultivation in each of two independent experiments, fish treated with 0,5 %
577 1g557 tended to have the highest mean weight and least size variance ($p > 0.05$), while the
578 number of external injuries was significantly ($p < 0.05$) reduced by more than a half when
579 compared with the non-supplemented control. Dietary supplementation of 1g557 showed
580 these beneficial effects for the tested fish sizes between 100 und 500 g when given at a
581 concentration of 0.5%. However, a higher 2.0% 1g557 supplementation reduced the fish
582 growth most probably due to the reduced amount of digestible energy inside the test feed.
583 Further studies need to address why the fish growth performed better and the incidence of
584 external injuries was drastically reduced under supplementation of the tested mixed layer
585 clay mineral, supporting our attempts to improve fish welfare through application of entirely
586 natural products under recirculation aquaculture conditions in future.

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594 **Author contributions**

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