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Potential of alginate, chitosan and polyethylene glycol as substances for colloidal drug delivery as determined by protein release and digestion

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ABSTRACT

Colloidal encapsulations can be applied as protective matrices in aquaculture feeds. They promise an ideal approach to protect bioactive substances such as oral vaccines, pre- or probiotics against degradation due to acidic environments or untimely lixiviation. Alginate, chitosan and polyethylene glycol (PEG) are substances frequently applied in encapsulations as protective matrices. However, essential information on their direct and comparable characteristics and their effects on digestion speeds after oral application in aquaculture are lacking. The current study evaluated in vitro release and retention profiles of a model protein bovine serum albumin (BSA) after encapsulation with four experimental formulations of protective matrices: ALG – alginate; AC –alginate and chitosan, AP - alginate and PEG and APC – alginate, PEG and chitosan. The iron marked treatment diets were fed to juvenile rainbow trout and digestion speed was investigated using radiographic imaging.

Digestion speeds did not differ significantly between treatments, with all test diets reaching the anterior fish intestine 10 h after feeding. The BSA retention under low pH was highest for the alginate-chitosan PM (84.7 ± 5.8 %). The inclusion of PEG reduced the retention rate in low pH but significantly increased the absolute BSA release. An oil coating significantly reduced the BSA release during the initial burst for the alginate, alginate-PEG and alginate-chitosan-PEG treatments and significantly reduced retention potential under neutral pH conditions. The feeding simulation trial showed that an oil-coated diet containing alginate-chitosan as a protective matrix can be used to protect the model protein during feeding (release to the water) and against the harmful milieu of the fish stomach. Different combinations of the investigated encapsulation substances can be used to achieve optimal encapsulation and protective characteristics depending on the application objective.

1. Introduction

Bioactive components find application in a multiplicity of disciplines such as human health, cosmetics, food production or animal health (Atanasov et al., 2015; Choudhury, 2023). Encapsulations present a quick and practical approach for efficient oral delivery of bioactive substances in aquaculture, where significant need exists for suitable solutions for bioactive delivery. Understanding and optimizing encapsulation methods and components is a key challenge for future development of orally applied aquaculture solutions in specialized feeds. Different encapsulation methods such as emulsions, liposomes or microgels are currently known to protect bioactive components against harmful digestive juices and degradation (Perry & McClements, 2020). While hard- or softshell capsules are used for delivery to terrestrial organisms. These have their limitation in aquaculture due to fish feeding behavior (Gullapalli & Mazzitelli, 2017).

The colloidal system is considered the most suitable for oral administration in aquaculture to protect the bioactive components such as probiotics, prebiotics, antibiotic or vaccines against degradation due to the harmful environment of the gastro-intestinal tract (GiT) and dissociating effect of water. Encapsulation techniques using alginate (Ghosh et al., 2015), chitosan (Alexakis et al., 1995; Tian et al., 2008) or PLGA (Fredriksen & Grip, 2012) showed promising results in terms of encapsulated active component efficiency and efficacy.

Several scientific papers describe alginate encapsulation: Ballesteros et al. (2015) demonstrated the efficacy of alginate by encapsulating an IHNV-targeting DNA vaccine for oral administration in rainbow trout, resulting in protective immune responses. Tian et al. (2008) used alginate to encapsulate a plasmid DNA for oral DNA-based immunotherapy in fish. Yu et al. (2019) developed alginate-chitosan coated nanoparticles for oral protein vaccine delivery, overcoming degradation challenges. Polk et al. (1994) used an approach with alginate and

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chitosan to produce microcapsules, offering a cost-effective oral delivery method for Vibrio bacterin and bovine serum albumin. Zhang et al. (2016) use calcium-alginate beads to encapsulate whey protein focusing on protein release and retention under different pH. Alexakis et al. (1995) microencapsulated calf thymus DNA in chitosan-coated alginate microspheres, demonstrating high encapsulation yield and controlled release. Mandal et al. (2006) used alginate to encapsulate *Lactobacillus casei*, which resulted in improved survival. Ghosh et al. (2015) employed alginate microcapsules for fish immunoprophylaxis, demonstrating structural robustness and controlled protein release of the delivered immunogen.

The encapsulation methods can be divided into spray application / dropping into $CaCl_2$ or water-in-oil emulsification. There is, however a large amount of methodological variation between previous studies, even those with the same encapsulation approach, therefore there is limited comparability between existing data. The use of alginate-chitosan as a protective barrier against the gastric destruction (pH 1.5) of the bioactive components in human applications was investigated by Yu et al. (2019). Thereby, the components were used as coating to protect the original bioactive-substance carrier (layered double hydroxide nanocomposites). When considering transfer of this method to aquaculture application, it appears this approach lacks sufficient protection against the initial release of the test-substance into the tank water. Especially for aquatic application, colloidal systems can provide extended protection for varying pH during animal administration. Wang et al. (2018) used colloidal alginate-chitosan microspheres to vaccinate channel catfish with a recombinant protein of *Streptococcus iniae*. Besides the release profile evaluation for pH 2 and pH 9 individually, a digestion stimulation of a 6 h initial release at pH 2 and a consecutive release at pH 9 was executed *in vitro*. The approach however ignored the momentous phase of initial pellet interaction with the rearing water at neutral pH. Still, the use of the micropellets increase the relative percent of survival (RPS) of fish from 35 % of unprotected vaccine to 60 % for the colloidal encapsulated vaccine by alginate-chitosan. Slightly increased RPS were achieved by the use of polyethylene glycol (PEG) as a protective matrix for the vaccination of juvenile rainbow trout against viral hemorrhagic septicemia (Adelmann et al., 2008). Before the PEG pellet was formed, salts were added to the PEG to later neutralize the acidic stomach environment of the orally vaccinated fish. The approach led to a RPS of 85 % in challenge tests, representing the huge potential of PEG as a protective matrix substance; even though no clear information about the release profile under the different pH conditions were available.

The aim of this study is to give insights into how the three encapsulation components alginate, chitosan and PEG affect the digestion rate in juvenile rainbow trout (*Oncorhynchus mykiss*), how these components affect the release rates of the encapsulated model-protein bovine serum albumin (BSA) under different pH conditions, and if an additional hydrophobic oil coating can reduce the initial burst of BSA.

2. Materials and methods

2.1. Protective matrix (PM) production

Each PM was produced by the water-in-oil emulsification method following the procedure modified from Ghosh et al. (2015) and Liu et al. (1997). A total of four PM treatments were formulated and produced:

ALG (with alginate)
AC (with alginate and chitosan)
AP (with alginate and polyethylene glycol)
APC (with alginate, polyethylene glycol and chitosan)

Briefly, for the aqueous phase of alginate PM (Treatment: ALG), 1 g medium-viscose sodium alginate and 100 mg bovine albumin serum (BSA) where dissolved in 100 ml distilled water. For the oil phase 15 m L Span-80 where added to 180 ml octane, under stirring with a shear mixer, the aqueous phase was introduced into the oil phase. After 90 s of stirring 15 ml Tween-80 were added and the mixture was adjusted to pH 3 using HCl. For micropellet hardening, under magnetic stirring an 8 % $CaCl_2$ solution (w/v) was added slowly. The solution was broken with 2-propanol for 25 min. Afterwards the micropellets were centrifuged and washed twice, frozen to -80 °C and lyophilized prior to experimental use. For the production of the alginate-chitosan pellet (AC), 0.5 % chitosan was added to the $CaCl_2$ solution and continued as described above. For the production of the PMs containing PEG (AP and APC) 10 % PEG_{1000} was added to the aqueous phase prior the emulsification process. The morphological appearance was evaluated by scanning electron microscopy. Therefore, the PM powder of the respective treatment was mounted on stubs, sputter coated with gold-palladium (Emscope SC500; Ashford, UK) and images were taken at 10 kV with a 10 mm working distance under a SEM (FEI Quanta FEG200; Eindhoven, the Netherlands).

2.2. Protective matrix (PM) digestion trial

For all experimental activities involving animals in the current research, the authors adhered to the prevailing ethical policies for animal methods and actions. Specifically, the animal trial was conducted in accordance with the German Animal Protection Act (TierSchG) and the regulations on the protection of animals used for experiments or other scientific purposes (TierSchVersV) in agreement with applicable EU directives and all procedures involving animals were approved by the Veterinary Authority of Bremen under the application administrative number TV148 (Animal Experimental Approval TV148).

2.2.1. Facility, fish and rearing conditions

The digestion trial was conducted at the Center for Aquaculture Research (ZAF) of the Alfred-Wegener-Institute Helmholtz-Centre for Marine and Polar Research, Bremerhaven in a recirculating aquaculture system (RAS). The RAS consisted of 16 individual adjustable glass tanks (48 × 38 × 49 cm, length x width x height), two cooling elements (Aquamedic Titan 2000), a foam sheet filter, a moving-bed nitrification bio-filter, a UV-sterilizer (Aquamedic Helix Max 36 W) and a monitoring system (Senect Filter Control with water level and pH/temperature sensor).

During acclimation and execution phase the water temperature was kept at 15 ± 1 °C, aeration and water flow was at 4.5 % min^{-1} and 350 L h^{-1}, respectively. Oxygen level was above 90 % throughout the experimental rearing.

A total of 240 juvenile rainbow trout, *Oncorhynchus mykiss*, Walbaum (14.7 ± 4.5 g) were obtained from a commercial fish farm (Die kleine Fischzucht, Geseke, Germany) and 15 fish were randomly distributed to the respective rearing tanks. For all treatments quadruplicates were used. Fish were acclimated to the experimental conditions (15 °C) for 10 days before the experimental feeding started. During acclimation, fish were hand-fed a commercial diet (F-1P Classic LT/F 2.5 mm, Skretting) twice daily until visual satiation. Pellet leftovers were siphoned to maintain high water quality.

2.2.2. Experimental diets

Three test diets ALG – alginate as PM, CHT – Chitosan as PM, PEG: Polyethylene glycol as PM and a control diet (control: CTR), each containing inert iron powder were tested. The diets were produced by the Technology Transfer Centre Bremerhaven (TTZ Bremerhaven) using a twin-screw cold extrusion system. The commercial diet (F-1P Classic LT/F 2.5 mm, Skretting) was used as basal pellet component. For the three test treatments, 8 g of crushed F-1P Classic LT/F was mixed with 0.6 g iron power plus 4 g PMs as outlined in 2.1 above. For the control treatment 12 g of crushed F-1P Classic LT/F was mixed with 0.6 g iron power. The mixtures were compressed three times to form stable 2.5 mm pellets.

2.2.3. Trial execution

To evaluate the different digestion rates of the four treatments, the 15 fish in each tank were fed at a feeding rate of 1 % (as determined by the total fish weight in each replicate). The time point of food addition was defined as t0. Over the first 10 h after t0, 1 fish / h was removed from each replicate (tank). This was followed by 6 h (three intervals) where 1 fish / 2 h / replicate was removed, followed by 8 h (2 intervals) where 1 fish / 4 h / replicate was removed. Thus, all 15 individual fish from each replicate were remove from the tanks over the total experimental period, which was thus 24 h post t0. After removal from the tank, fish were anesthetized with MS-222, killed by a sharp blow to the head and body weight was noted. Euthanized animals were kept at 5 °C until the radiographic evaluation was finished.

2.2.4. Radiographic evaluation

Radiographic images of the chilled animals were taken at Hanover University of Veterinary Medicine Foundation, Department of Fish Diseases and Fish Husbandry. After the images were taken, fish were stored at -80 °C in case any further analysis or verifications were needed.

The intestinal tract was subdivided into 7 sections in the radiographic images (Fig. 1), following the definition of Weinreb and Bilstad (1955). Assessment of pellet position was differentiated to the included iron particles in the test diets (Fig. 3). In case of unclear evaluation via radiographic images, the respective stored animal was defrosted and pellet position determined by dissection.

2.3. PM protein release trials

2.3.1. Trial 1 pH dependency and trial 2 oil coating effects

To evaluate BSA retention / release of the different PMs under laboratory conditions, 10 mg of the respective lyophilized PM (see Section 2.1) was added to reaction tubes (Eppendorf Safe-Lock Tubes) in triplicate. After preparation of all sample tubes, 1.9 mL of H_2O (pH 3 or pH 8) was added to each test tube and tubes were gently rotated (test-tube-rotator 34528, Snijders Scientific). After 15 min, 30 min, 12 h and each full hour tubes were centrifuged (10 min, 16,000 g) and samples were taken and stored at 7 °C until protein determination. For the oil coating trial, 50 µl fish oil (provided by the TTZ, Bremerhaven) was added to the test tube prior the water addition. The PM / oil mixtures were left for 5 min for the oil to soak all the PM.

2.3.2. Trial 3 simulated PM digestion

The simulation trial was carried out to investigate BSA release of the different PM formulations in varying pH milieus. The feeding process was simulated as follows: 1 Pellet feeding – introducing to fish tanks with exposure to system water at pH8. 2 Pellet ingestion – stomach with exposure at pH3. 3 Pass-through to anterior intestine with exposure at pH8 and 4 Pass-through to posterior intestine with exposure at pH 8.

For each treatment, 10 ± 0.3 mg of coated and uncoated PM was filled into the sample tubes (Eppendorf Safe-Lock Tubes). Oil-coated treatments contained pellets coated with 30 µl oil. The experiment was carried out in quadruplicate. After the oil-coating process was finished, (1) 1 ml of pH8 water was added to all samples and placed on the rotator. After 5 min, tubes were centrifuged (10 min, 16,000 g) and supernatant was removed, labeled and stored for protein determination. The same procedure was repeated with (2) 1 ml of ph3 water and 50 min reaction time, (3) 1 ml of pH 8 water and 50 min reaction time and (4) pH 8 water and 5:50 hour reaction time on the same PM probe. When all samples were collected, protein concentration was determined.

2.4. Protein determination

Protein concentration of the stored samples (7 °C) were determined with the Pierce™ Coomassie protein-assay-kit (Thermo Scientific™) following the manufacturer's instructions.

2.5. Statistical analysis

Data from protein release trials were tested for normality and homogeneity of variance. Dependent on the distribution and heteroscedasticity a two-way-ANOVA or GLM (Method=inverse.Gaussian) was performed. The TukeyHSD or Holm-Sidak test was used to show differences between and within the treatments. A significance level of $p < 0.05$ was used for all tests.

Retention rate = 100 – protein concentration pH8 / protein concentration pH3 * 100

3. Results

3.1. Digestion rate analysis

The PM digestion trial showed that $a \approx 30$ % dietary inclusion of alginate, PEG or chitosan had no significant effect on digestion rates of juvenile rainbow trout. After 16 h, all pellets reached the posterior intestine / rectum. The passage into the anterior intestine (Fig. 2, GiS E) was reached after 10–12 h in all diet formulations. The fastest digestion rate (non-significant) in the intestinal tract (GiS D-G) was present in the control treatment which had no inclusion of any PM test substances.

3.2. pH dependency of basal PM

3.2.1. Effects of different formulations

The relative release rates of the different PM treatments differ significantly between the initial burst (IB) ($t = 0.5$ h) and final release potential (RP) ($t = 24$ h | $F(1,32) = 39.02$, $p < 0.001$), the different PM formulations ($F(3,32) = 55.8$, $p < 0.001$) and the two pH conditions (pH3 and pH8 | $F(1,32) = 1037$, $p < 0.001$), (Fig. 4).

After the IB phase (t0.25–1 h) a positive trend in relative BSA release was present in the ALG, AP and APC treatment. Only the AC treatment showed a reduction of free BSA in the condition media (pH 3/8 water)

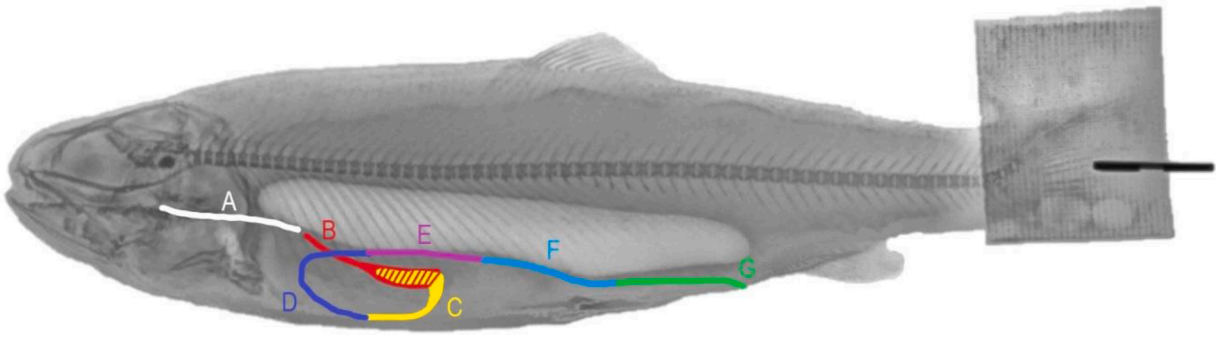

Fig. 1. Radiographic image of an juvenile rainbow trout. The intestine sectioned were divided as follow: A – esophagus and stomach, B – Stomach, C – Stomach and pyloric caeca, D – pyloric caeca and anterior intestine, E – Anterior intestine, F – mid intestine, G – posterior intestine / rectum.

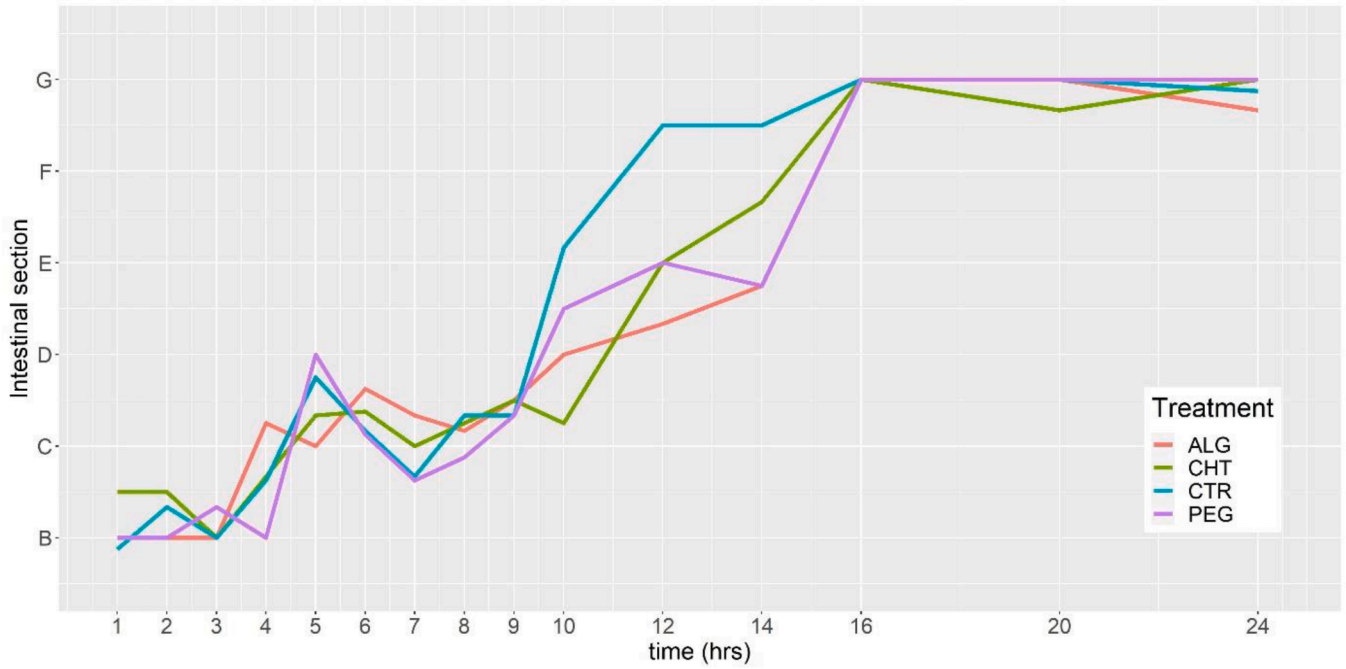

Fig. 2:. Intestinal passage rate of juvenile rainbow trout. Fish intestine was divided into following sections (GiS): A – esophagus and stomach. B – Stomach. C – Stomach and pyloric caeca. D – pyloric caeca and anterior intestine. E – Anterior intestine. F – mid intestine. G – posterior intestine / rectum. Four different feed formulations were used, containing a commercial diet as main component (approx. 66 %) and the test substance (33 %): ALG – alginate PM, CHT – Chitosan PM, PEG: Polyethylene-glycol PM or not test substance (control: CTR).

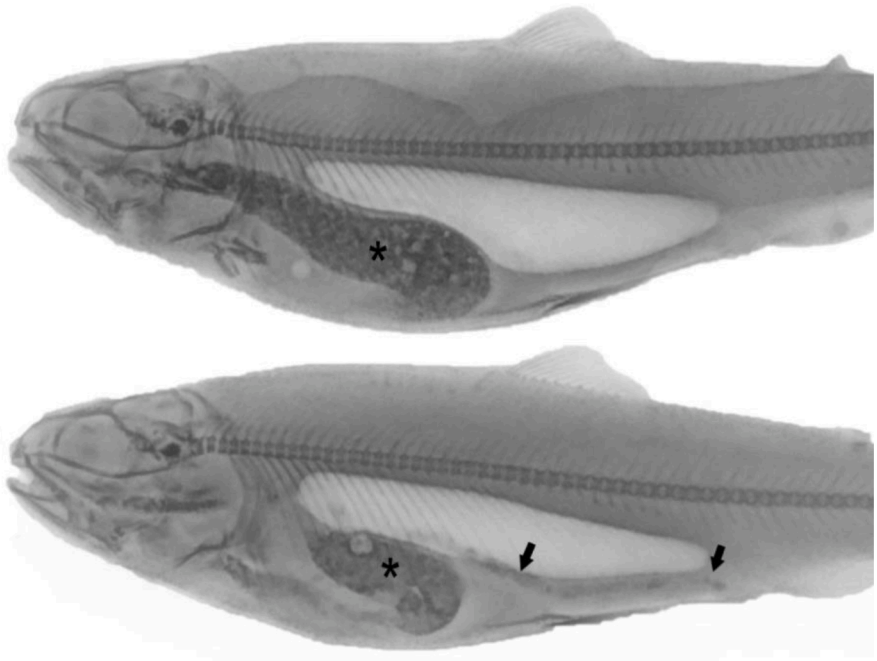

Fig. 3. Inverted radiographic image of juvenile rainbow trout post feeding with the iron marked treatment. The upper image was taken 1 h post feeding. Iron marked feed is visible in the gut (asterisk). The lower picture shows a fish 20 h post feeding. The iron marked feed is still present in the stomach (asterisk) and mid- and posterior part of the intestine (arrow).

for the first 8 h. No significant increase in relative BSA release was observed (GLM, inverse.gaussian $p = 0.8$) after IB and RP was handled as final release potential / rate. Highest retention rates were present in the AC and ALG formulations with 84.7 ± 5.8 % and 75.7 ± 3.7 %, respectively, followed by the AP formulation with 57.8 ± 2.1 % and APC formulation with 35.3 ± 4.3 %.

Besides the relative release rate, the absolute values differed significantly across the pH conditions and PM formulations (3-way-ANOVA $F(3,32) = 34.6$, $p < 0.001$). The lowest BSA loading was present in the AC treatment and highest in the PM formulations with PEG inclusion (Table 1).

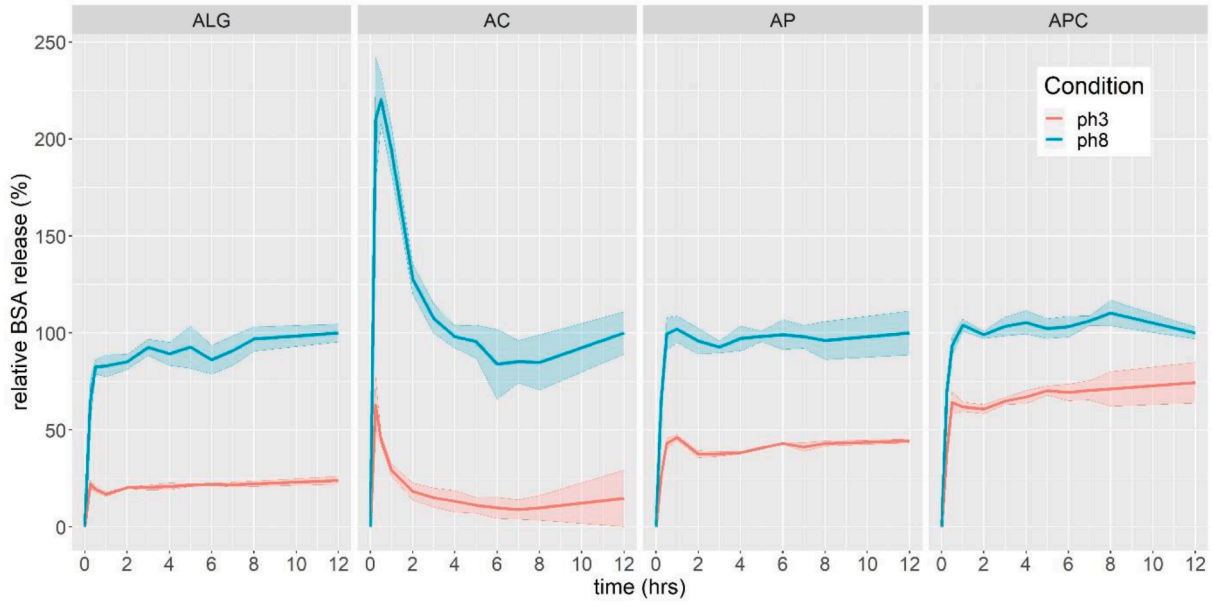

Fig. 4. Relative release profile of basal formulations under different pH conditions (ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene glycol and APC - PM with alginate, polyethylene glycol and chitosan).Each sub-graph shows the two pH conditions for each treatment. Sample means are given by the centered line. the area presents the respective standard deviation, $n = 4$.

Table 1
Summary of Initial burst rate (IB) and release potential (RP) for the four treatments (ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene glycol and APC - PM with alginate, polyethylene glycol and chitosan) under the two tested release conditions. Values present the absolute release values in mg BSA / g PM ± *S*.D. Differences were tested for significance by a 2-way-ANOVA (AOV). The Holm-Sidak method was used to present differences within the conditions. A significance level of $\alpha < 0.01$ was used. $N = 3$.

	Condition	ALG	AC	AP	APC	ANOVA results
IB	pH3	17.4	7.9 ±	70.5 ±	112.7 ±	F(3,8) =
RP	pH8	± 1.4a	0.4a	4.7b	10.2c	222.3 $p <$
	pH3	74.1	38.5	163 ±	163.9 ±	0.001
	pH8	± 3.3a	± 2.2b	13.8c	8.4c	F(3,8)=174.5
		21.6	2.6 ±	72.7 ±	130.5 ±	$p < 0.001$
		± 1.8a	2.6b	1.8c	18.4d	F(3,8) =
		89.8	17.5	163.8 ±	175.4 ±	112.1 $p <$
		± 4.2a	± 1.9b	18.5c	5.3c	0.001
						F(3,8) =
						164.6 $p <$
						0.001

Table 2
Retention ratios of the oil coating trial for the initial burst (IB) and release potential (RP). ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene glycol and APC - PM with alginate, polyethylene glycol and chitosan. Values present percentages ± *s*.d. Differences were tested for significance with a 2-way-ANOVA (AOV). The Holm-Sidak method was used to present differences within the conditions. A significance level of $\alpha <$ 0.01 was used. $N = 3$.

	Coating	ALG	AC	AP	APC	ANOVA results
IB	-	82.6 ±	38.5 ±	163 ±	163.9 ±	F(3,8) =
RP	Oil	5.9^a	2.2^b	13.8^c	1.3^c	174.5, $p <$
	-	20.2 ±	29 ±	26.5 ±	20.2 ±	0.001
	Oil	1	2.1	2.1	7.1	F(3,8) = 4, $p =$
		89.8 ±	17.5 ±	163.8 ±	175.4 ±	0.05
		4.2^a	1.9^b	18.5^c	5.3^c	F(3,8) =
		35.6 ±	6 ±	30.4 ±	25.2 ±	164.6, $p <$
		1.4^a	0.4^b	1.7^c	1.3^d	0.001
						F(3,8) =
						286.2, $p <$
						0.001

3.3. Effects of oil coating as hydrophobic barrier

The PM processing with fish oil as hydrophobic coating led to significant differences in release profiles among the treatments (3-way AOV F(3,32) = 38.9, $p < 0.01$). While the release rates were not significantly reduced in the ALG, AP and APC treatment, the release in the oil coated AC was significantly higher ($t = 11.7, p < 0.01$) during IB. For all treatments but AC, the BSA release for the initial burst *coated PM was less than that of uncoated PM* (Table 2).

Absolute release profiles showed strongly reduced release rate at IB and RP (3-way-ANOVA$_{\text{treat x coating}}$ F(3,32) = 278.7, $p<0.001$). The retention rate (RR) of the oil treatment differed significantly between the different PM formulations ($\chi^2 = 36.3$, df = 3, $p < 0.001$). Significant differences within the different PM treatments were found between APC – AC ($q = 7.2, p < 0.05$), APC – ALG ($q = 7, p < 0.05$), AP-AC ($q = 4.2, p < 0.05$) and AP-ALG ($q = 4.2, p < 0.05$). PM formulations with the inclusion of chitosan showed an increased initial burst when directly compared to the ALG and AP treatment. In both of these formulations (ALG and AP) the initial BSA release peak as present in the uncoated formulations (Fig. 5, blue lines) was reduced and a continuous release of BSA was observed.

3.4. Digestion stimulation

Besides the significant differences among the different coated and uncoated PM formulations for each pH condition (Fig. 2), the oil coating led to significantly different BSA releases for the same PM formulation. Oil coating led to significantly reduced BSA release in tank condition for AC-PM formulation (Holm-Sidak $t = 5.2\ p < 0.001$) and a consequential significantly higher BSA release of the oil coated AC-PM formulation in the stomach (Holm-Sidak $t = 26.4\ p < 0.001$), anterior intestine (Holm-Sidak $t = 7.3\ p < 0.001$) and posterior intestine (Holm-Sidak $t = 5.5\ p < 0.001$) pH conditions. Further, the oil coating significantly decreased the release in the ALG-PM formulation (Holm-Sidak $t = 3.5\ p < 0.01$) in the anterior intestine pH condition and significantly increased the BSA release for the AP-PM formulation (Holm-Sidak $t = 3.1\ p < 0.01$) in the

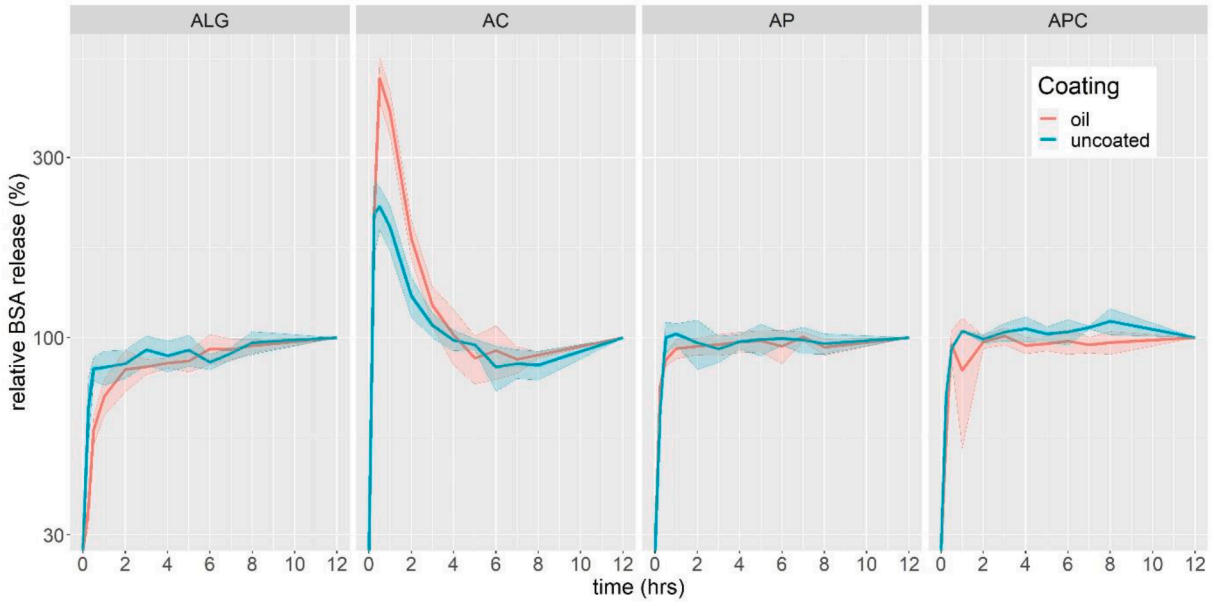

Fig. 5. Relative release profile of basal and oil coated PM formulations under pH 8 conditions (ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene glycol and APC - PM with alginate, polyethylene glycol and chitosan).Each facet shows the coated and uncoated PM for the respective treatment. Sample means are given by the centered line. The area presents the respective standard deviation. The y-axis is giving in a logarithmic scale $n = 4$.

stomach pH condition. Although the AC treatment showed the lowest cumulative absolute release of BSA, the absolute BSA release in the organism simulation (stomach, anterior & posterior intestine) was the highest among all treatments for the coated and uncoated AC treatment (Table 3).

3.5. Morphological appearance of micropellets

The use of alginate or alginate with chitosan led to the formation of circular micropellets.

The inclusion of PEG to the micropellet formulation led to a deformation of the circular micropellets (Fig. 7). Micropellets without the use of PEG showed less adherence to each other. Fig. 7

4. Discussion

Encapsulations or protective matrices (PMs) are key to effective delivery of bioactive components in aquaculture specialized diets or oral vaccinations. Understanding protection, digestion and release of bioactives under application of various PMs is key to choosing and applying the right PM in specialized diets to obtain optimal bioactive protection and delivery. In the following study, multiple proposed and applied PMs were compared in controlled feeding experiments and laboratory release experiments applying different pH conditions reflecting the digestive system/milieu and with different pellet coatings. The results provide clear indications for optimization of coatings as related to the water and gut milieu, along with timing and completeness of bioactive component release. Digestion times were unaffected by matrices and the highest release protection in low pH conditions and best release rates under simulated conditions were achieved by the AC treatment with alginate and chitosan as PM. The additional oil coating significantly reduced all absolute release rates for the tested PMs.

In rainbow trout, the gastric pH is influenced by the stomach filling after feeding and strongly acidic (pH below 3) stomach milieu is only present prior to a meal or 48 h after a meal or later (Bucking & Wood, 2009). Successful targeted administration of active components require a homogeneous uptake or a minimum pellet uptake for each individual animal. In juvenile rainbow trout, feeding a single dose without a starvation period improves uptake homogeneity and minimum pellet uptake (Just, Köllner and Slater, 2021).

Based on this knowledge, a modification of protective mechanism should be directed to reduce the loss of bioactive component to the system water as this implies a loss of ≈ 98 ± 2 % of BSA loading as present in this study for the uncoated PMs (Fig. 6).

Further, the stability of the bioactive pellet plays a significant role in uptake and pass- through the stomach into the GiT. Better attraction, pellet uptake and pass-through are known for pellets with low disintegration stability (Bogevik et al., 2021) and the inclusion of PEG into the pellet could be beneficial as it increases the "water attraction" and consequently increases the soaking of a pellet. Different apparent properties which were noted during the handling as distinct, definable spheres were validated by the microscopic investigation. Due to the less distinct and round spheres for the control and AC treatments, showed the PMs with inclusion of PEG increased agglomerations of the lyophilized PM and resuspended pellets. The digestion trial showed that the inclusion of the test PMs into a commercial pellet did not differ the

Table 3
Absolute BSA release for water release and organic uptake locations (stomach. anterior and posterior intestine). RR – Retention rate, RA – Release advantage of coated treatments. ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene glycol and APC - PM with alginate, polyethylene glycol and chitosan. Differences were tested for significance with a 1-way-ANOVA. The Holm-Sidak method was used to present differences within the treatments. A significance level of $\alpha < 0.05$ was used.

Location	Coating	ALG	AP	AC	APC	ANOVA
Initial burst (System water)	uncoat	138.3 ± 8.4 [a]	270 ± 11.9 [b]	87.5 ± 11.8 [c]	285.9 ± 6 [d]	$F(3,12) = 393.6$ $p < 0.001$
	coated	90.3 ± 14.1 [a]	255.3 ± 19.6 [bd]	32.6 ± 5.5 [c]	257.4 ± 18.4 [d]	$F(3,12) = 222.4$ $p < 0.001$
	RR (%)	34.7	5.5	62.8	10.0	
Organism release	uncoat	2.4 ± 0.9	1.9 ± 0.9	4.7 ± 1.6 [a]	1.3 ± 0.1	$F(3,12) = 7.8$ $p < 0.01$
	coated	2.9 ± 2.6 [a]	12 ± 6 [b]	20.4 ± 1.8 [c]	4.9 ± 2.3 [a,d]	$F(3,12) = 19.8$ $p < 0.001$
	RA (%)	21.4	526.6	336.1	280.6	

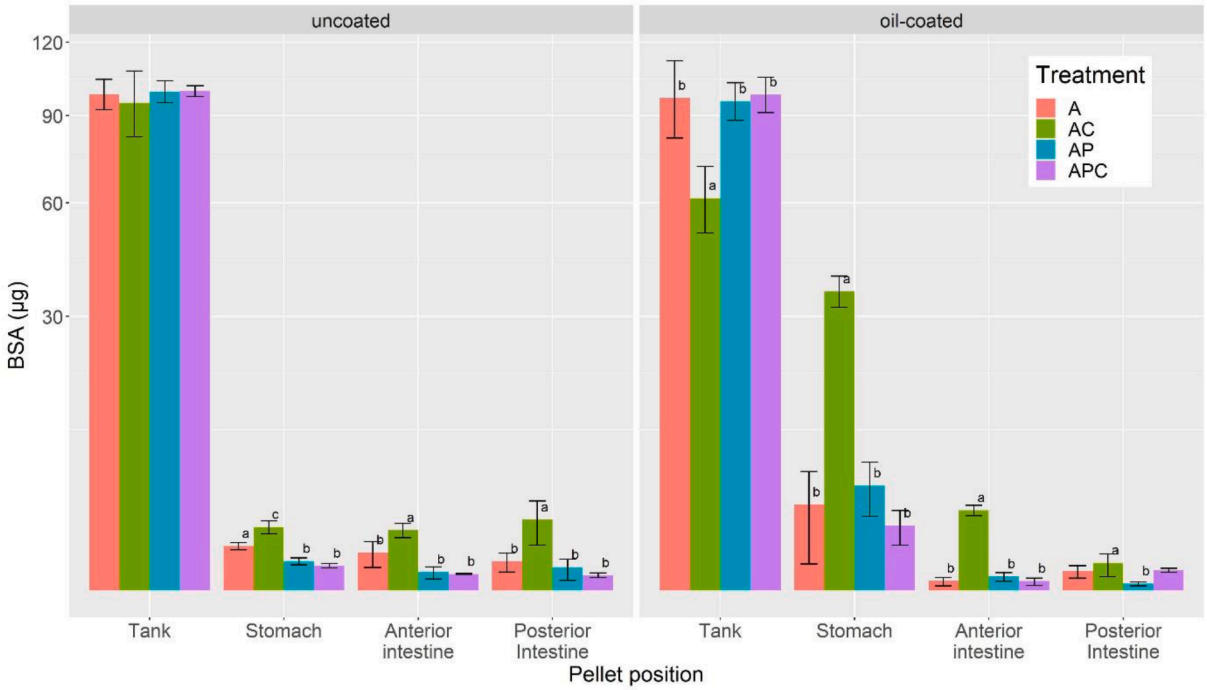

Fig. 6. BSA release during intake and digestion simulation. Data were normalized to 100 µg / treatment. Data were tested position-wise with an ANOVA. When data were significantly different, the Tukey HSD test was used to show differences between the different PM formulations, ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene glycol and APC - PM with alginate, polyethylene glycol and chitosan. A significance level of $\alpha < 0.05$ was used. y-axis is presenting a square-root scale.

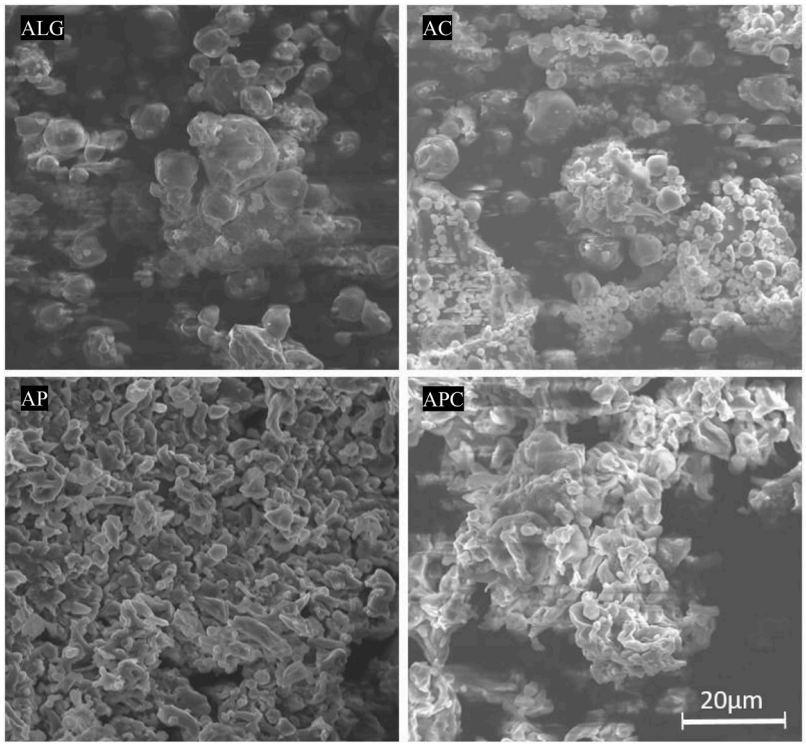

Fig. 7. Morphological appearance of lyophilized micropellets. ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene glycol and APC - PM with alginate, polyethylene glycol and chitosan Magnification: 3000x.

digestion speeds significantly. The anterior and posterior intestine were reached 10 h and 16 h post feeding respectively for all formulations (Fig. 2). Expected increasing digestion speed based on the laxative effects of PEG (Di Palma et al., 2002) were not found in this study.

When attempting oral application of fragile and bioactive components, protective mechanisms such as encapsulation should be effective for at least 4 h to move the bioactive component safely and unharmed through the system water and stomach into the intestine. Coating diet

pellets with a hydrophobic substance such as fish oil can protect the encapsulation matrix, as in this study with its main component alginate. Coatings decrease the initial burst in the system water and consequently increase the amount of encapsulated substance entering the target species. Oral vaccination by an uncoated alginate encapsulated vaccine was performed by Ballesteros et al. (2015) and significantly higher amounts of vaccine were needed compared to injective vaccination to induce an adequate immune response. Based on the results of this study it is very likely that vaccination trials with the inclusion of PEG to the oral vaccine formulation and a hydrophobic top-coating will increase the efficiency of such vaccination trials.

Alginate can interfere with the polar group of BSA, leading to interaction with the encapsulation materials. Fundamentally, amphiphilic molecules can react with the polar groups to form stable foams, emulsions or suspensions (Dickinson, 1999). Blocher McTigue and Perry (2019) evaluated the different electrostatic effect of BSA and showed coacervation with encapsulation polymers and presents the main dependency between pH and encapsulation success. The PEG used in this study for the encapsulation process has been utilized as surfactant to increase the water – oil interactions in previous studies, however beneficial effects on intestinal uptake of the target bioactive molecules are expected, as PEG can alter the barrier function of the epithelial tissue (D'souza & Shegokar, 2016).

Reduced BSA loading in the chitosan containing PM formulations in this study might be based on the positive electrical potential of chitosan in comparison to alginate (Perry & McClements, 2020). In the encapsulation process with the inclusion of chitosan, low pH was used to form microcapsules that have higher retention potential at low pH (Liu et al., 1997), whereas higher encapsulation efficiency was achieved when the encapsulation media was set to pH 8 (McTigue & Perry, 2019). This inverse dependency may explain the significantly reduced absolute BSA release rates in the alginate- chitosan treatments in all trials. During the production process, the acidic chitosan solution may negatively interfere with the positive charged BSA at low pH. At a pH below 5.5 the BSA charge will become positive and thereby repels from the positive charged chitosan. The mechanism of alginate repelling under different pH has been demonstrated by Zhang et al. (2016). The inclusion of chitosan in this study led to the highest initial BSA burst and at the same time to the highest BSA release in the simulated fish intestine. The addition of PEG to the alginate-chitosan treatment reduced the beneficial effects of an oil coating, compared to the non-chitosan treatments (Fig. 5).

The previously described initial burst of BSA into the condition media was present for all PM formulations. From the different basal encapsulation methods: alginate, alginate-chitosan, alginate-PEG and the combination of both showed that under unchanged production methods, the omission of chitosan and the inclusion of PEG and top oil coating will increase the total release while improving the retention rate against tank water and the gastric juices of the fish stomach during feeding.

Further studies should focus on the effect of different encapsulation sizes with modified surface – volume ratio to reduce the unwanted initial burst. As described by Polk et al. (1994) significantly reduced BSA release rates by the use of chitosan did not apply for nanocapsules (diameter $\approx$ 5 nm).

The radiographic method applied in this paper provided a quick and direct tool to determine the digestion times of different feeds. With regard to the application and administration of an oral vaccine or other bioactive substance, the exact determination of the location of the respective substance in the animal is of major importance. Protective matrices should be specifically modified based on the results of this trial, to guarantee an unharmed passage of bioactives through the stomach into the intestine of rainbow trout in aquaculture applications. The method offers a precise determination of gastric, mid- and posterior intestine contents, however the precision to determine progress along the intestine for the anterior part (Fig. 1, GiS: D and E) was hindered, due to the overlapping loop structure of the gastrointestinal tract (GiT, Fig. 1). The radiographic method was first described by Molnár and Tölg (1960) with iron powder by Talbot and Higgins (1983). The advantages of this non-invasive method (Talbot & Higgins, 1983) could be further used and modified for a live digestion evaluation based on improved technology and rearing techniques. To increase the precision of pellet determination and therefor minimize uncertainties, increased iron powder grain size is also recommended.

5. Conclusion

The substances tested for the encapsulation of bioactive substances as applied in this study have high potential to increase the efficiency and success of those bioactive substances for oral application. In contrast to encapsulation methods for human or other terrestrial animal application, oral use in aquatic application is linked to protection against the initial burst in the system water. Based on the results of the current study, chitosan in combination with alginate and a hydrophobic oil coating thus presents the best method to increase the bioactive component release into the target species. Even though the inclusion of PEG decreased the release profile quality of the model-protein BSA, higher total releases were present and potential beneficial interactions with the intestinal epithelial cells are expected and should be investigated in future research. The radiographic evaluation method should be further modified to improve precision. Nonetheless, it can be used for live and euthanized fish species and varying sizes to determine not only digestion speeds of modified or new feedstock and feed additives, but also to determine residual times for the intestinal areas of choice.

Ethical approval

The authors followed all ethical policies of the journal in all methods and actions. The animal trial was conducted in accordance with the German Animal Protection Act (TierSchG) and the regulations on the protection of animals used for experiments or other scientific purposes (TierSchVersV) in agreement with applicable EU directives and were approved by the Veterinary Authority of Bremen under the administrative number TV148.

CRediT authorship contribution statement

Philip N. Just: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Matthew J. Slater:** Conceptualization, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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