THE GEORGE WASHINGTON UNIVERSITY
WASHINGTON, DC

School of Engineering & Applied Science

Mechanical & Aerospace Engineering
Science and Engineering Hall
800 22nd Street, NW, Suite 3000
Washington, DC 20052

t 202-994-6749
f 202-994-0127
maeng@gwu.edu

May 27, 2020

Dr. Vineeta Bajaj,
Review Editor
JoVE (Journal of Visualized Experiments)
17 Sellers St.
Cambridge, MA 02139

Dear Dr. Bajaj,

We are submitting the revised manuscript titled "Macro-rheology characterization of gill raker mucus in the Silver Carp, *Hypophthalmichthys molitrix*," toward your consideration for publication in JoVE. In this revision, we addressed your comments and suggestions using "Track changes". Our detailed responses are also attached with this letter.

We look forward to hearing back from you regarding our manuscript.

Thank you for all your suggestions, as they improved the quality of our revised manuscript greatly.

.

Sincerely,

Kartik V. Bulusu, D.Sc.
Assistant Research Professor
Department of Mechanical and Aerospace Engineering
The George Washington University, Washington DC 20052
Phone: (202) 491 1084
Email: bulusu@gwmail.gwu.edu

Attachment: [Response to editorial comments]

<u>Response to editorial comments</u>

We thank the review editor for the suggestions and comments presented in the current review of our manuscript. Below we responded point-by-point to all the suggestions and comments made in the review. The editor's suggestions are marked in '**Times New Roman font**' *and our response follow in 'Calibri* font'. *The instances where text from the revised manuscript is included verbatim in this rebuttal, we present them using* 'Times New Roman font' *in our response. The revised manuscript has been put together with "Track Changes".*

1. **The manuscript needs a thorough proofreading.**

   Response:
   Thank you for suggesting a thorough proof-read in this revision. We made sure that there are no typographical and formatting errors in this revision.

2. **Abstract is made crisp, please check.**

   Response:
   We are grateful for the changes made in our abstract.

3. **Please remove the redundancy make the introduction crisp. This actually needs a good copyediting.**

   Response:

   We thank the review editor for the guideline on improving the introduction-section. We modified this section following the review editors' comment. Several typographical errors were fixed in this revision. The changes can be noticed in the revised manuscript using "Track changes". We took care to avoid redundancy by deleting repetitive words and phrases in this section. Further, any verbatim mention of sentences across the manuscript have been avoided. Complex sentences were broken down into small phrases so that the section can be made crisp and clear. We took care in reordering the references so that they are pointing to the citations accurately. The figures were also reordered to make sure they are correctly described in the revised text.

   Responses to the editor's specific comments are presented below:

   a. ***"The GR mucus properties investigated in the protocol using a strain rate controlled, rotational rheometer follow."*** **Follow what ?**

      Response:

      The sentence was modified to address the editor's concerns as presented below.

"The GR mucus properties investigated in the protocol using a strain rate controlled, rotational rheometer are described below."

b. ***"Increase in apparent viscosity would lead to a reduction in velocity of flowing liquid for any give pressure gradient?"***

Response:

The sentence was modified to address the editor's concerns, but we eventually deleted it as it was not relevant for describing Figures 3A and 3B.

c. ***"The GR mucus properties investigated in the protocol using a strain rate controlled, rotational rheometer follow."*** **Follow what? Test are performed to generate data pertaining to G' G' and n? Please revise and make crisp sentences throughout.**

Response:

The sentence was modified to address the editor's concerns as presented below.

"The types of rheometer tests performed to monitor data pertaining to storage modulus (G'), loss modulus (G") and apparent viscosity (η) are described below. The dynamic oscillation tests (strain sweeps and frequency sweeps) monitored G' and G" under controlled oscillation of cone geometry."

The following text has been included in the modified introduction-section:

"The silver carp, *Hypophthalmichthys molitrix*, is a planktivorous filter feeder and an invasive species that has infiltrated several natural waterways in the United States. This species was initially introduced in the upper Mississippi River basin to control algal blooms[1-3]. The silver carp is an extremely efficient feeder. Typically, its consumable food particle sizes range from 4 to 20 µm to larger zooplankton that are around 80 µm[3-5]. This species has outcompeted other native fish and can potentially cause enormous damage to native waterways by limiting available resources[1-2,6]. Thus, filter feeding fish such as the silver carp and the bighead carp pose a major threat to the Great Lakes[1-2,6-8].

Filter feeding fish possess special organs called the gill rakers (GRs) with a thin layer of mucus residing on their surface. These organs improve the efficiency of filtration and aggregation of small particles from the incoming fluid. The goal of the protocol presented herein is to characterize the non-Newtonian, shear thinning material property and yield stress of the GR mucus acquired from the inner surface of the gill rakers in the silver carp. The value of yield stress of the GR-mucus, ascertained using a rotational rheometer, is of interest. The measured yield stress is referred to as an "apparent yield stress" since it depends on the testing methods such as steady shear rate- or dynamic oscillatory strain-type[9-10]. Currently the most utilized rheological phenomenon is the shear-thinning 'yield-stress fluid,' or the transition from solid-like to liquid-like behavior at a critical applied stress[9,11]. The apparent yield stress is the minimum shear stress required to initiate flow or that at which irreversible plastic deformation is first observed when the mucus transitions from a gel-like material to a fluid-like material. This behavior can be observed in structured viscoelastic materials such as the mucus layer on GRs. The transition from gel-like to fluid-like behavior of the GR mucus entails two functions i.e., an adhesive role to gather food particulates and a transport vehicle role to assist in the delivery and filtration process. The extended function of the mucus includes creating diffusion barriers in disease resistance and respiration, providing controlled release of nutritional factors, toxic components and excretion, metabolic pathways for feeding and nesting, predator protection, and boundary layer modification that improves the locomotion and propulsive efficiency[12-14].

Complex fluids like the mucus possess properties that vary with flow conditions and require additional measurement parameters to define their bulk scale physical behavior. To monitor the viscosity and yield stress of GR mucus, rheological measurements are performed using a rotational rheometer. The rotational rheometer applies a steady or oscillatory shear stress or strain by means of a rotating disk in contact with the fluid sample and measures its response. The rationale behind using this instrument and technique is that the rheometer can provide a set of measurements to describe the material properties of the GR mucus of the silver carp, which cannot be defined by viscosity alone.

The mucus is a viscoelastic material and its mechanical response to an imposed deformation is between that of a pure solid (governed by Hooke's law of elasticity) and that of a pure liquid (governed by Newton's law of viscosity)[15-16]. The complex macromolecular network contained within the mucus can stretch and reorient in response to external forces or deformation. A rotational rheometer is comprised of a cone geometry and a Peltier plate as shown in **Figures 1** and **2** (see **Table 1** for instrumentation specifications). The objective of this study was to develop a protocol to determine the rheological properties of the GR mucus. An advantage of the rotational rheometer over a viscometer is its ability to make dynamic measurements using small sample volumes. The GR mucus sample volume in this study was approximately 1.4 mL. The viscometer, on the other hand, is limited to constant shear rates and requires large sample volumes.

The rheological properties of the mucus are expected to vary greatly within the silver carp anatomy. For example, the rheological properties of the mucus residing on the inner GR surfaces may be different from that on the outer GR surfaces or the epibranchial organ. To account for the potential variability of mucus properties in different regions of the fish, the acquired GR mucus sample was diluted and solutions of various concentrations were created to develop an understanding of the variation in rheology. The rotational rheometer with cone geometry and a Peltier plate was then used in this protocol to investigate the various small samples of mucus. The data and results regarding mucus rheology reported after executing the protocol demonstrated the efficacy of the measurement technique and were not meant to be generalized across the entire silver carp population. The protocol presented herein can be extended to investigate mucus rheology across larger sample sets to test other hypotheses.

The purpose of this study is to demonstrate the variation of rheological properties of GR mucus rheology with three different mucus concentrations (400 mg/mL, 200 mg/mL and 100 mg/mL). The 400 mg/mL concentration represents the raw mucus sample harvested from the fish GRs. Distilled water (DI) was used to dilute the raw mucus sample into 200 mg/mL and 100 mg/mL concentrations. Diluting the mucus samples allowed us to evaluate the extent of shear thinning and apparent yield stress as a function of concentration and to determine the concentration at which the GR mucus transitions to non-Newtonian behavior. A shaker was used to break down any large clumps of mucus in the samples to mitigate errors in the rheological data due to inhomogeneity.

In most vertebrates, including fish, the predominant mucus-forming macromolecules are glycoproteins (mucins) that tend to swell in water by entanglements or chemical cross-linking and create a gel-like material[12-13,17-20]. The high-molecular-weight gel-forming macromolecules and high-water content reflects the slipperiness in the mucus[13]. A high degree of inter-macromolecular interactions leads to gel-formation whereas lower levels of inter-macromolecular interactions or broken bonds result in high-viscosity fluids[21].

The processes of food particulate filtration in filter feeding fish are aided by GR mucus-related properties such as cohesion and viscosity that determine its potential toward adhesion and tack [22]. The strength of mucus-based adhesion depends on specific intermolecular interactions, electrostatic or hydrophobic interactions[23]. Sanderson et al.[24] conducted a suspension-feeding study in blackfish wherein they found the evidence for mucus-based adhesion. They stated that the adhesion of suspended food particulates with a mucosal surface is followed by the transport of aggregated clumps of particles bound together with mucus by directed water-flow acting on it[24]. Endoscopic techniques were used to observe filtered particles[24]. The mucus exposed to shear strain rates generated from water-flow facilitates the delivery of food particulates to digestive organs.

Literature on the range of shear rates and practical limits in the rheological testing of GR mucus is scarce. Therefore, guidance was sought from rheological studies on gastric, nasal, cervical and lung mucus, salmon skin mucus, hagfish slime, and bone-joint surface lubricant wherein the rheological characterization and non-Newtonian attributes of the mucus has been ascertained previously[11-12,25-31]. More recently, the effect of fish skin mucus on locomotion and propulsive efficiency has been studied using constant shear rate viscometry. Skin mucus rheology studies (without any dilution or homogenization) pertaining to seabream, sea bass and meagre demonstrated non-Newtonian behavior at typically low shear rates[14]. In another related study, the raw skin mucus samples from dorsal and ventral sides of the Senegalese sole were found to exhibit non-Newtonian behavior, indicating a higher viscosity of the ventral mucus at all shear rates considered[32].

Other rheological protocols pertaining to hydrogel scaffold development and for highly concentrated suspensions using a constant shear rate viscometer have also been reported in the literature[33-34].

The GR mucus properties investigated in the protocol using a strain rate controlled, rotational rheometer are described below. These properties describe the rheology of complex biological fluids [25]. For Newtonian fluids, the apparent viscosity remains constant and is shear-rate-independent and the shear stresses vary linearly with shear strain rates (**Figures 3A and 3B**). For non-Newtonian fluids (such as shear-thinning fluids) viscosity is shear-rate-dependent or deformation-history-dependent (**Figure 3A and 3B**). The loss modulus (G") represents the extent to which the material resists the tendency to flow and is representative of fluid viscosity (**Figure 4**). The storage modulus (G') represents the tendency of the material to recover its original shape following stress-induced deformation and is equivalent to elasticity (**Figure 4**). The phase angle (δ) or loss tangent value, is calculated from the inverse tangent of G"/G'. It represents the balance between energy loss and storage and is also a common parameter for characterizing viscoelastic materials ($\delta = 0°$ for a Hookean solid; $\delta = 90°$ for a viscous liquid; $\delta < 45°$ for a viscoelastic solid and $\delta > 45°$ for a viscoelastic liquid) (**Figure 4**) [25]. The apparent yield stress (σ_y) in structured fluids represents a change of state that can be observed in rheological data from steady state sweep and dynamic stress-strain sweeps[10]. If the external applied stress is less than the apparent yield stress, the material will deform elastically. When the stress exceeds the apparent yield stress (marked as "average stress" in **Figure 3B**), the material will transition from elastic to plastic deformation and begin to flow in its liquid state[35]. Measuring the storage modulus (G') and loss modulus (G") in the mucus-sample under oscillatory stress (or strain) conditions quantifies the change in the material state from gel-like to viscoelastic liquid-like behavior.

The types of rheometer tests performed to monitor data pertaining to storage modulus (G'), loss modulus (G") and apparent viscosity (η) are described below. The dynamic oscillation tests (strain sweeps and frequency sweeps) monitored G' and G" under controlled oscillation of cone geometry. The dynamic strain sweep tests determined the linear viscoelastic region (LVR) of the mucus by monitoring the intrinsic material response (**Figure 4**). Strain sweeps were used to determine the yielding behavior at constant oscillation frequency and temperature. The dynamic frequency sweep tests monitored the material response to increasing frequency (rate of deformation) at a constant amplitude (strain or stress) and temperature. Strain was maintained in the linear viscoelastic region (LVR) for the dynamic frequency sweep tests. The steady-state shear rate tests monitored the apparent viscosity (η) under steady rotation of the cone geometry. The GR mucus was subjected to incremental stress steps and apparent viscosity (η, Pa.s) was monitored for varying shear rate ($\dot{\gamma}$, 1/s).

The protocol presented in this paper treats the GR mucus as a complex structured material of unknown viscoelastic nature with a certain linear viscoelastic response range.

The fish mucus was extracted from the GRs of the silver carp during a fishing expedition at the Hart creek location in the Missouri River[1-2,36]. An array of GRs inside the mouth of a Silver carp is shown in **Figure 5A** and a schematic drawing of the same is presented in **Figure 5B**. An excised GR is shown in **Figure 5C.** Extraction of mucus from GRs of the silver carp is summarized in the schematic drawings, **Figures 5D,E**. All the rheometer tests were performed under a constant, controlled temperature of 22 ± 0.002 °C, the temperature recorded at the fishing site[1-2,36]. Each mucus sample was tested three times with the rheometer, and the averaged results are presented in figures along with the statistical uncertainty bars."

4. **Please move all the softwares to the table of materials as these are commercial. [Comment on the note pertaining to protocol step-2]**

   Response:

   Thank you for mentioning the need for moving commercial nomenclature. We have now moved the commercial software information into the table of materials as suggested. We also combined tables 2 and 3, into one table of materials following the comment #8 below.

5. **"Perform these steps on the samples individually." Added here, please check. [Comment on protocol step-2.8]**

   Response:

   Thanks for the suggestion. We updated the sentence to make sure it fits our protocol step as presented below.

   "Perform these steps on the available mucus concentration samples individually."

6. **Steps 2.8.13 – 2.8.15 – Redundant, please remove**

   Response:

   Thank you for mentioning the redundancy of steps 2.8.13 – 2.8.15. We have removed them from the manuscript.

7. **[Comment on the discussion-section first paragraph]** ***"Our objective in using this technique and protocol is to establish that it is well-suited for further investigation with larger mucus sample volumes. We acknowledge that more samples from a school of silver carp would need to be analyzed to fully characterize the rheological properties of the GR mucus and the data presented herein is not a generalization across the silver carp population. Our technique is justified due its efficacy with rheological characterization of mucus when small volumes are available."*** **This can be moved to the discussion.**

Response:

Thank you for the suggestion to move the sentences to the discussion-section. We agree that it fits better in the discussion-section and followed the suggestion. These sentences are now the first paragraph of the discussion-section. The updated discussion-section is presented as our response to comment #10 below.

8. **FIGURE AND TABLE LEGENDS – Chemicals, instruments, and reagents, buffers, etc should be combined into one table of materials. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.**

Response:

Table 2 and 3 from the previous revisions are combined into one table of materials as suggested in comment #4 and #8. We updated the table of materials with the information to the best of our knowledge and hope that they are now looking complete.

a. **Table 2: List of Chemical Reagents Used – Table 2 is not the chemical reagents used?**

Response:

We hope that the updated table of materials can make things clearer. Thank you for the suggestion.

9. ***"Supplementary Figure 5: Gap Options Page. The gap tab is used to set how the rheometer will zero the distance between the geometry and plate. Specifically, an axial force of 1N will be used to gage where zero distance is***." **- ????**

Response:

The caption and the description are modified to make the Supplementary Figure 5 clear.

"Supplementary Figure 5: Measurement Gap Options The **"**gap" tab options are accessed to set the conditions for zero gap mode and traverse velocity of the measurement head. An axial contact force between the geometry and the Peltier plate was set to 1 Newton to ensure the zero-gap reference, i.e., the contact between cone geometry and the surface of the Peltier plate. The measurement head can then be made to accurately traverse to the measurement gap of 28 µm between the 40 mm 1^{o} cone geometry and the Peltier plate."

10. DISCUSSION – Please make the discussion crisp and remove redundancy throughout.

Response:

We thank the review editor for the guideline on improving the discussion-section. We modified this section following the review editors' comment. The first paragraph from the Representative results-section was moved as suggested in Comment #7. The portions that were redundant or repetitive after the results-section were removed.

The critical steps within the protocol, a modification of the protocol, suggested improvements, limitations and significance of the protocol are presented in separate paragraphs to ensure that the audience can peruse through them.

We discussed the physical insights from the GR mucus rheology data by reviewing schematic drawings presented in the Figures 3 and 4, so that the audience can easily understand the data trends and incorporate the approach presented in our protocol in future experiments.

One of the main points we made in this section is the need to review data that can be unsuitable for extended analysis. We felt this is an important point when working with very small sample volumes like the GR mucus and complex biological fluids. We cited papers by Nelson and Ewoldt (2017) and Ewoldt et al. (2015) that discuss instrumentation limitations, low-torque effects and secondary flow effects in detail.

The data we presented by the successful execution of the protocol were of high fidelity. The future users of this protocol can benefit from the discussion. The following text has been included in the modified discussion-section:

"One of the main objectives of developing this protocol is to establish that it is well-suited for rheological characterization of GR mucus when very small sample volumes are available. We acknowledge that more samples from a school of silver carp would need to be analyzed to fully characterize the rheological properties of the GR mucus and the data presented herein are not a generalization across the silver carp population. Our technique is justified due its efficacy with rheological characterization of small sample volumes and can be used in extended investigations with larger mucus sample volumes***.***

The critical steps within the protocol are the preparation of mucus solutions of various concentrations, measurements and data acquisition using a rotational rheometer, and graphical representation and data analysis for physical insights.

Physical insights into GR mucus data are drawn from schematic representations shown in Figures 3 and 4, that are annotated with attributes of the expected material behavior. **Zero-shear strain rate viscosity** (η_o) values can be observed at low-shear strain rates where mobility of the material molecules dominates (Figures 3A and 8A). **Infinite-shear strain viscosity** (η_∞) values in non-Newtonian fluids are orders of magnitudes lower than the zero-shear strain rate viscosity. These data can be noticed at high shear rates where there is little or no dependence on intermolecular interactions (Figures 3A and 8A). For non-Newtonian fluids, apparent viscosities progressively decrease as the shear rates increase and attain a constant low value (Figures 3A and 8A). **Yielding behavior in the GR mucus under steady state measurements** can be represented with slope as shown in Figure 3A and presented in Equation 1., where η_a represents the apparent viscosity, σ_y is the (constant) yield stress and $\dot{\gamma}$ is the shear strain rate.

$$\eta_a = \frac{\sigma_y}{\dot{\gamma}} \tag{1}$$

Figures 3A and 8B are presented on a log-log scale and therefore, Equation 1 attains the following form:

$$log\, \eta_a = k - \log \dot{\gamma} \tag{2}$$

where k – represents the apparent yield stress. On a log-log scale, the apparent viscosity decreases with a slope of '-1' indicating material yield as shown in Figure 3A [10]. The 200 mg/mL and 400 mg/mL mucus concentrations possessed slopes of -1.8 and -0.91, respectively, and demonstrate yielding behavior (Figure 8A). Under dynamic oscillation measurements, the viscoelastic characteristics are independent of the strain amplitude in the Linear Viscoelastic Region (LVR) (Figure 4). The **yielding behavior in the GR mucus under dynamic oscillation measurements** can be observed as the viscoelastic material (GR mucus) enters the non-linear viscoelastic region (NLVR) as the storage modulus (G') decreases (Figure 4). In the NLVR regime the viscoelastic material will demonstrate solid-gel-like behavior if the storage modulus is greater than the loss modulus (G' > G"). When the loss modulus exceeds the storage modulus (G' < G"), a "crossover" between G' and G" data occur. As shown in Figures 7B-C, the 200 mg/mL and 400 mg/mL GR mucus concentrations demonstrated fluid-like behavior marked by the "crossover" between G' and G" data. The **apparent yield stress under steady state measurements** is represented as the average value of stress until an inflection point is reached (Figure 3B). Thereafter, the stress begins to increase sharply with an increase in the shear strain rate as shown in Figures 3B and 8B. The GR mucus data (200 mg/mL and 400 mg/mL concentrations) showed shear-thinning fluid behavior until the material begins to yield (Figure 8A and 8B). The apparent yield stress was observed clearly in 200 mg/mL and 400 mg/mL mucus concentrations due to their non-Newtonian characteristics (Figures 8B). The **apparent yield stress under dynamic oscillation measurements** are shown in Figures 4 and 7A-C as the "crossover" region between G' and G" data, followed by G" values exceeding G'. The 400 mg/mL GR mucus data showed shear-thinning, non-Newtonian behavior. The onset point of material yield was observed with an apparent yield stress of approximately 0.2736 Pa (Figure 7C). The **hydrogel-to-fluid like transition** with phase angle ($\delta = \tan^{-1}$ (G"/G')) changes are presented in Figures 4 and 7D-F. The extrema in the phase angle is associated with a Hookean solid at 0^o and viscous fluid at 90^o as shown in Figure 4. The phase angle values around 45^o were attributed to transition of gel-like behavior of the material to a fluid-like behavior. The 400 mg/mL mucus concentration clearly showed a change in the material characteristic from hydrogel to fluid like behavior through the process of yielding with an apparent yield stress of ~ 0.2736 Pa (Figure 7F).

Understanding the measurement limitations and avoiding data unsuitable for physical interpretation is a challenge with complex and soft biological fluids, especially when working with small sample volumes[11]**.** The data generated under low-torque and secondary flow effects are unsuitable for physical interpretation and are dependent on the geometry used in the rheometer (such as cone and plate in this study). These regimes were identified to avoid any misrepresentation of experimental data suffering from instrument resolution and measurement artifacts due to momentum diffusion. **Low-torque limits** (Figures 5B and 8A) are functions of geometry and minimum torque generated by the instrument (Table 1). Under steady shear measurement conditions, the criterion for rejecting data affected by the low-torque limit for a cone-plate geometry of radius (R) with minimum torque (T_{min} = 10 x 10^{-9} Nm, Table 1) has been discussed by Ewoldt et al., (2015) and is presented below [11]:

$$\eta > \frac{\left(\frac{3}{2\pi R^3}\right) T_{min}}{\dot{\gamma}} \tag{3}$$

where $\dot{\gamma}$ is the shear strain rate. The regimes of instrumentation limitation governed by low-torque and secondary flow effects are marked in Figures 6A and 8A. Unlike the 100 mg/mL GR mucus concentrations, the 200 mg/ml and 400 mg/mL GR mucus concentrations were unaffected by low-torque effects clearly demonstrate non-Newtonian, shear thinning behavior with high zero-shear strain rate viscosities at low shear strain rates. The criterion for minimum measurable viscoelastic moduli under dynamic oscillation measurements has been discussed by Ewoldt et al**.,** (2015) and presented below (Equation 4) [11]**.** In Equation 4, for a cone-plate geometry of radius (R) the minimum torque under oscillatory shear (T_{min} = 2 x 10^{-9} Nm, Table 1).

$$G_{min} = \frac{\left(\frac{3}{2\pi R^3}\right) T_{min}}{\gamma_0} \tag{4}$$

where G_{min} is the storage modulus (G') or loss modulus (G") and γ_0 is the shear strain rate. The regimes of instrumentation limitation governed by low-torque effects are marked in Figures 6A and 6B. The **secondary flow regime under steady state measurements** is governed by an inward momentum diffusion of the fluid by means of an eddy residing within the rotational cone and plate geometry[11]**.** The secondary flow pattern increases torque incorrectly making the fluid appear to be shear-thickening (Figure 8A). The secondary flow limit in Figure 8A was drawn using the following relation:

$$\eta > \frac{L^3/R}{Re_{crit}} \rho \dot{\gamma} \tag{5}$$

where L = βR, β is the cone angle, R is the cone radius, ρ = 1000 kg m^{-3}, Re_{crit} = 4 and $\dot{\gamma}$ is the shear rate. This regime helped in the accurately estimating the infinite-shear strain viscosity (η_∞) values in GR mucus samples.

A modification of the protocol can be made by using a flat-plate geometry instead of the cone-plate geometry as shown in protocol presented herein. The flat-plate tests should be performed with a parametric variation of the measurement gap in the rotational rheometer to reveal the dependence of apparent yield stress on the measurement gap and geometry. The **suggested improvements** of the protocol presented in this paper are described below. A parametric variation of the strain amplitude in the linear viscoelastic regime (LVR) and oscillation frequency should be performed. 'Tack and peel' rheology tests should be performed to develop a full understanding of the adhesivity of the GR mucus. Rheology characteristics of GR mucus should be performed on larger sample volume ensembles along with studies to measure any traces of blood cells to account of its effect on the overall GR rheological properties.

The limitations of the protocol are described below. The intricacies of the GR mucus extraction-procedures and the presence of blood cells or tissue fragments in the mucus samples may influence the rheology of the mucus. However, it should be noted the mucus used in the protocol did not have any visible traces of blood. The GR mucus sample is a heterogenous material and can possess different rheological properties due to the variance in location of and the conditions post-extraction. This limitation was addressed by sufficiently homogenizing the GR mucus using a shaker to breakdown any large clumps of mucus and tissue presence. Another important limitation is the very small GR mucus sample volumes (approximately 1.4 mL) that can be harvested for analyses that constrain a generalization of GR mucus properties.

The significance of this protocol is that it allows for an accurate rheological characterization of non-Newtonian, biological fluids such as the mucus. The protocol presented herein paves the way for investigating other similar biological fluids associated with human, animal and plant secretions. In addition, synthetic fluids or polymer-based solutions that are analogs of biological fluids can be testing using this protocol to understand material properties under varying stresses, oscillation frequencies, and temperature. The protocol is well-suited for rheological characterization of biological fluids when very small sample volumes are made available."

11. The text mentions about 36 citations, please check. Please also see that citation number and references corresponds to each other.

Response:

Thank you for pointing this out. The missing reference was identified and included in the revised manuscript. Due the nature of changes made in the introduction- and discussionsections the citation numbering of the references were also checked and we feel that they are now complete.

The full list of 36 references are included below:

1. Cohen, K.E., Hernandez, L.P. The complex trophic anatomy of silver carp, *Hypophthalmichthys molitrix*, highlighting a novel type of epibranchial organ. *Journal of Morphology*. 279,1615–1628, (2018).
2. Cohen, K.E., Hernandez, L.P. Making a master filterer: Ontogeny of specialized filtering plates in silver carp (*Hypophthalmichthys molitrix*). *Journal of Morphology.* 279,925–935., (2018).
3. Cremer, M. and Smitherman, R. Food habits and growth of silver and bighead carp in cages and ponds. *Aquaculture*. 20(1), 57–64, (1980).
4. Battonyai, I. et al. Relationship between gill raker morphology and feeding habits of hybrid bigheaded carps (*Hypophthalmichthys spp.*). Knowledge and Management of Aquatic Ecosystems. 416,36, (2015).
5. Zhou, Q., Xie, P., Xu, J., Ke, Z., Guo, L. Growth and food availability of silver and bighead carps: Evidence from stable isotope and gut content analysis. Aquaculture Research. 40(14), 1616–1625, (2009).
6. Freedman, J. A., Butler, S. E., Wahl, D. H. Impacts of invasive Asian carps on native food webs (Final Report). Urbana-Champaign, IL: University of Illinois, Kaskaskia Biological Station. (2012).
7. Nico, L., Fuller, P., Li, J. Silver carp (*Hypophthalmichthys molitrix*)—FactSheet. (2017).
8. Walleser, L., Howard, D., Sandheinrich, M., Gaikowski, M., Amberg, J. Confocal microscopy as a useful approach to describe gill rakers of Asian species of carp and native filter-feeding fishes of the upper Mississippi River system. *Journal of Fish Biology*. 85(5), 1777– 1784 (2014).
9. Nelson, A. Z., Ewoldt, R. H. Design of yield-stress fluids: a rheology-to-structure inverse problem. *Soft Matter*. 13, 7578—7594, (2017).
10. Chen, T. Rheological Techniques for Yield Stress Analysis. TA Instruments Applications Note, RH025 (2020).
11. Ewoldt, R.H., Johnston, M.T., Caretta, L.M. Experimental challenges of shear rheology: how to avoid bad data. in: S. Spagnolie (Editor), Complex Fluids in Biological Systems, Springer, (2015).
12. Thornton, D. J., Sheehan, J. K. From Mucins to Mucus: Toward a more coherent understanding of this essential barrier. *Proceedings of the American Thoracic Society*. Vol 1, 54 – 61, (2004).
13. Shepard, K.L. Functions for fish mucus. *Reviews in Fish Biology and Fisheries*. 4, 401-429, (1994).
14. Fernández-Alacid, L., et al. Skin mucus metabolites in response to physiological challenges: A valuable non-invasive method to study teleost marine species. *Science of the Total Environment.* 644, 1323-1335, (2018).

15. Wagner, C.E., Wheeler, K.M., Ribbeck, K. Mucins and Their Role in Shaping the Functions of Mucus Barriers. *Annu. Rev. Cell Dev. Biol.* 34,189–215, (2018).

16. Bird, R.B., Armstrong, R.C., Hassager O. *Dynamics of Polymeric Liquids, Volume 1: Fluid Mechanics*. New York: Wiley. 1,255–84, (1987). Silberberg, A. Mucus glycoprotein, its biophysical and gel forming properties. *Symp. Soc. Exp. Biol.* 43, 43-64, (1989).

17. Mantle, M., Allen, A. Isolation and characterisation of the native glycoprotein from pig small intestinal mucus. *Biochem. J.* 195, 267-75, (1981).

18. Allen, A., Hutton, D.A., Pearson, J.P., Sellers, L.A. Mucus glycoprotein structure, gel formation and gastrointestinal mucus function. In Nugent, J. and O'Connor, M., eds. *Mucus and Mucosa* (Ciba Foundation Symposium). London: Pitman, 137-56, (1984).

19. Asakawa, M. Histochemical studies of the mucus on the epidermis of eel, *Anguillajaponica. Bull. Jap. Soc. Scient. Fish.* 36, 83-7, (1970).

20. Fletcher, T.C., Jones, R., Reid, L. Identification of glycoproteins in goblet cells of epidermis and gill of plaice *(Pleuroneces platessa* L.), flounder *(Platichthys flesus* (L.)) and rainbow trout (Salmo *gairdneri* Richardson). *Histochemical* J. 8, 597-608, (1976).

21. Silberberg, A. Mucus glycoprotein, its biophysical and gel forming properties. *Symp. Soc. Exp. Biol.* 43, 43-64, (1989).

22. Hills, B. *The Biology of Surfactants.* Cambridge: Cambridge Univ. Press. 408, (1988).

23. Aubert, H., Brook, A.J., Shephard, K.L. Measurement of the adhesion of a desmid to a substrate. *Br. Phycol.* J. 24, 293-5, (1989).

24. Sanderson, S.L., Cech, J.J., Patterson, M.R. Fluid dynamics in suspension feeding black fish. *Science.* 251, 1346-8, (1991).

25. Lai, S.K., Wang, Y.Y., Wirtz, D., Hanes, J. Micro- and macrorheology of mucus. *Advanced Drug Delivery Reviews*. 61(2), 86-100, DOI: 10.1016/j.addr.2008.09.012, (2009).

26. Chaudhary, G., Ewoldt, R.H., Thiffeault, J.L. Unravelling hagfish slime. *J. R. Soc. Interface.* 16, (2019).

27. Downing, S., Salo, W., Spitzer, R., Koch, E. The hagfish slime gland: a model system for studying the biology of mucus. *Science.* 214, 1143–1145, (1981).

28. Hwang, S.H., Litt, M., Forsman, W.C. Rheological properties of mucus. *Rheologica Acta*, Band 8, Heft 4, (1969).

29. Litt, M. Mucus rheology. *Arch Intern Med.* 126, 417 – 423, (1970).

30. Quarishi, M.S., Jones, N.S., Mason, J. The rheology of nasal mucus: a review. *Clin. Otolaryngol.* 23, 403 – 413, (1998).

31. Nordgård, C.T., Draget, K.I, Seternes, T. Rheology of salmon skin mucus. *Annual Transactions - The Nordic Rheology Society.* 23, (2015).

32. Fernández-Alacid, L., et al. Comparison between properties of dorsal and ventral skin mucus in Senegalese sole: Response to an acute stress. *Aquaculture*. 513, (2019).

33. Yüce, C., Willenbacher, N. Challenges in Rheological Characterization of Highly Concentrated Suspensions — A Case Study for Screen-printing Silver Pastes. *J. Vis. Exp.* 122, (2017).

34. Sultan, S., and Mathew, A. P. 3D Printed Porous Cellulose Nanocomposite Hydrogel Scaffolds. *J. Vis. Exp.* 146, (2019).

35. Barnes, H.A., Hutton, J. F., Walters, K. *An Introduction to Rheology*. Amsterdam: Elsevier, (1989).
36. "National Water Information System." *USGS Current Conditions for USGS 06910450 Missouri River at Jefferson City, MO*, U.S. Geological Survey, https://nwis.waterdata.usgs.gov/usa/nwis/uv/?cb_00010=on&cb_00060=on&cb_00065=on&format=gif_default&site_no=06910450&period=&begin_date=2018-09-19&end_date=2018-09-21.

We sincerely hope that the review editor will find the revisions made in the manuscript in agreement with the suggestions and that this work is meritorious for publication in the Journal of Visualized Experiments.