

Comments on revised MS water-155158 version 2

First of all: this is an interesting and possibly promising study. However, it deserves a more thorough and critical approach. Publication is therefore considered premature.

Although several comments of the authors are thoughtful and make sense, the major criticism of this reviewer has not been addressed satisfactorily. The data presented and the conclusions based on these data, characterised as not-justified by this reviewer, have not or not satisfactorily been changed. Only a few comments have been added to the manuscript and a conclusion was changed.

-In the abstract the authors present the use of mucus for determining stress parameters as novel, and they present the significant increase of glucose and cortisol in stressed fish as new findings. This is already known for a long time: these results are conformatory. The main conclusion of this study should focus on the new elements of this study: the other parameters measured.

-Comments 2, 3: Unfortunately, the values of 12 out of 15 of these parameters do not show a statistically significant difference between plasma and mucus, and do not convincingly support the claims of the authors. They seem to argue that an overall, more general approach of the data by correlation analysis compensates the absence of statistical significant differences of the individual parameters. However, the reverse is more likely. For instance, Spearman's correlation coefficient assumes linear data associations (in other words: it determines to what degree the data fit a straight line). The time courses of several parameters are not linear. Pearson's will consider the ten-fold increase of cortisol as an outlier. That the method is robust against outliers implies here that the tenfold increase of cortisol will be ignored, whereas it is the most significant stress effect of the whole experiment. The inadequacy of Spearman for this type of data is clearly illustrated in the example mentioned in the first referee report: the positive correlation of cortisol with EA. Whereas cortisol shows five- to tenfold increases, EA hardly shows any change. The relatively high (and difficult to explain) correlation has probably been increased further by the high variability of the data. The result is: unfounded and even false conclusions. (Whereas the authors state in their comment 1 that the Spearman correlation analysis has been eliminated, it is still present in version 2.)

-Standard deviations were very high and most of the parameters tested did not show statistically significant differences when tested individually. The standard deviations were very high. An important point of criticism was that the methods had not been rigorously tested for sensitivity, specificity and reproducibility. This was considered important because these assay characteristics may change in mucus as a chemically very complex matrix. The authors have added information on the methods obtained from literature. Apparently these methods have not been tested in mucus as matrix. They now admit that these methods require further investigation. However, critical validation of the methods should always take place before the experiments and before publication of the results.

-The authors further state in their reply that they aimed at discerning a battery of stress biomarkers in both plasma and mucus. That aim is relevant, but a distinct description of that battery is missing and the criteria for the selection of parameters for that battery remain unclear.

-Comment 9: The earlier criticism did not concern the method (PCA) but the lack of detailed information about the procedures and the lack of clear conclusions. Only general conclusions are still drawn: These data ...spot out the drawbacks of just determining plasma analytes..", and: "It highlights the usefulness of skin mucus as a complementary matrix..". Why? Please be more specific and use (convincing) arguments.

-The general conclusion that “stress has a generalized negative effect in fish affecting multiple systems” is raises questions. That the effects observed are negative for the fish is incorrect: Some may reflect damage to the fish (effects of the stressor, not of stress) and some may be positive (the effects of the stress response, especially in its early phases). And, finally, whether the effects were positive or negative was not part of this study, and thus cannot be a general conclusion of this study.