Flash Flood simulation and valve behavior of Mytilus galloprovincialis measured with Hall sensors

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Abstract

Mussels close their shell as a protective strategy and the quantification of this behavioral marker may represent an alarm signal when they are exposed to environmental stressors. In the present study, we investigated the ability of the Mediterranean mussel *Mytilus galloprovincialis* to recover and then the resilience or inertia of valve activity after a pulsing exposition to diverse levels of salinity (5, 10, 20 and 35 PSU as reference value). The trial simulated an event of drastic and sudden reduction of seawater salinity thus mimicking an event of Flash Flood from intense rain. Valve gaping and movements were measured in continuous cycle for ten days using a customized magneto-electric device which uses Hall sensors. Results showed that under normal conditions of salinity (35 PSU) the general pattern of valve movements was a continuously open state with sporadic spikes indicating a closing motion. At salinity of 5 PSU mussels reacted by closing their valves, leading to a 77% mortality on the fourth day. At salinity of 10 PSU animals were observed with closed valves for the entire duration of the exposure and no mortality occurred, they showed a significant reduction in the valve activity once the reference value of salinity was re-established. In contrast, salinity of 20 PSU did not trigger a significant behavioral response. Interestingly, there no define rhythms of valve movements were recorded during salinity challenges.

Key words Mussels, *Mytilus galloprovincialis,* Valve activity, Hall sensor, Salinity

INTRODUCTION

Mussels are powerful bio-indicators commonly utilized to monitor spatial distributions and temporal trends of chemical pollutants in coastal and estuarine regions (Goldberg 1975; Goldberg 1978; Viarengo & Canesi 1991; Pavičić *et al.* 1993; Cajaraville *et al.* 2000; Petrović *et al.* 2001; Klarić *et al.* 2004; Jakšić *et al.* 2005; Hamer *et al.* 2008) and more recently to assess changes in the health status of the marine ecosystem in response to climate change (Zippay & Helmuth 2012; Caza *et al.* 2016). Their use is largely based on assessment of changes in the animal's body composition, which is only possible after the animals are collected and sacrificed for analyses of soft parts (Goldberg 1978).

Among behavioural markers, mussel valve movement is widely recognized as an integrative measure of physiological functions and useful in biological early warning systems (BEWSs), including the Mosselmonitor© (de Zwart *et al.* 1995) and the Dreissena-Monitor© (Borcherding 2006). Mussel valve movements are related to vital activities such as respiration, feeding, excretion, and circadian rhythms, which can change under stressful environmental conditions (Rao 1954; Langton 1977; Ameyaw-Akumfi & Naylor 1987; Fujii & Toda 1991; Gnyubkin 2010). Mussels also open and close their valves in a defensive reaction to external stimuli such as touching or shading, the sudden approach of a predator, as well as in response to a deteriorating environment. For example, toxic red tides, oxygen deficiency, low salinity, or elevated water temperatures have been shown to induce abnormal valve gap (Dharmaraj 1983; Gainey & Shumway 1988; Baldwin & Kramer 1994; de Zwart *et al.* 1995; Rajagopal *et al.* 1997; Kramer & Foekema 2000; Kramer 2009; Dowd & Somero 2013). Therefore, quantifying valve movements (i.e. recurrence of opening and closure of shell) and gaping (i.e., the distance between two valves of the shell) under a variety of natural and experimental conditions can aid in understanding the general physiological responses of these organisms to abiotic stresses in the environment (Burnett *et al.* 2013; Beggel & Geist 2015; Lummer *et al.* 2016), biotic interactions (Rovero *et al.* 1999; Rovero *et al.* 2000), and exposure to toxins (Halldórsson *et al.* 2008; Redmond *et al.* 2017).

Conventional methods of measuring valve movements include kymographic and strain-gauge methods (Kuwatani 1963; Fujii 1977; Higgins 1980), electromyography (Jenner *et al.* 1989), impedance electrodes (Tran *et al.* 2004), laser sensors (Redmond *et al.*

2017) and magneto-electric devices (Kramer & Foekema 2000; Wilson *et al.* 2005; Robson *et al.* 2007).

Magneto-electric devices assess the valve movements in the form of the output voltage from the Hall element sensor (attached to one shell valve) generated by changes in the external magnetic field from a magnet attached to the other valve. Such technology has been used to study valve gape behavior in pearl oysters, *Pinctada fucata*, in the early detection of noxious dinoflagellate blooms (Nagai *et al.* 2006). The Hall sensor system was tested in the blue mussel, *Mytilus edulis*, when exposed to diverse levels of predation (Robson *et al.* 2007), and later to study gaping and pumping behaviors in the endangered freshwater bivalve *Margaritifera margaritifera*, the bay mussel *Mytilus trossulus*, the scallop *Pecten maximus,* and the cockle *Cerastoderma edule* (Robson *et al.* 2010). Hall sensor technologies were also used to evaluate the filtration behavior in freshwater mussels to evaluate the effect of de-icing salt (NaCl) in *Anodonta anatina* (Hartmann, Beggel, Auerswald, Stoeckle, *et al.* 2016) and the effect of fine sediment concentration in *Unio pictorum* (Lummer *et al.* 2016). Magneto electric devices have been applied in the Mediterranean mussel *Mytilus galloprovincialis* (Lamarck 1819) only once for studying the effect of circadian rhythms on valve movements (Gnyubkin 2010).

Even though the measurements of mussels' valve's activity with different methods have received much focus, many authors demand extensive effort to develop advanced data processing and interpretation to ameliorate the quality of threshold of disturbance of environmental stressors including climate stressors (Bae & Park 2014; Beggel & Geist 2015; Hasler *et al.* 2017; Redmond *et al.* 2017).

Coastal systems are particularly exposed by a variety of human and climatic drivers, for instance: changes in sea level rise (SLR), sea surface temperatures, ocean acidity and extreme (weather) events. The concept of extreme events are split into three categories: (i) weather and climate variables (temperature, precipitation, winds); (ii) phenomena related to weather and climate extremes (monsoons, El Niño and other modes of variability, [extra-] tropical cyclones); and (iii) impacts on the physical environment (extreme sea level rise, droughts, and flash floods) (Seggel & De Young 2016). Current data (Seggel & De Young 2016) suggest an increase in the frequency and intensity of flood hazards in the Mediterranean ecoregions increasing the vulnerability of transitional waters, coastal lagoons and aquaculture facilities in coastal areas.

Flash floods are considered one of the most important stressors for mussels, an actual threat both for natural mussel beds and mussel farming (Hamer *et al.* 2008; Polsenaere *et al.* 2017). For instance, in mid-November 2013, the Cyclone Cleopatra, while hitting the coasts of north Sardinia (W Mediterranean, Italy), poured almost 18 inches of rain in less than two hours (corresponding to up to six months of rain in the same region in normal years). A second drastic event occurred in October 2018 in south Sardinia with 14 inches of rain in less of 20 hours. These flash floods events caused a mass mortality (90-100% of loss in the production) of the mussels reared in these areas (Santa Gilla Lagoon, and Gulf of Olbia), which represents the most traditional areas for mussels' farming in Italy (Niedda *et al.* 2015; Turolla 2016).

In a *time* perspective "early warning signals" based on mussel valve gaping recorded in discrete locations (i.e. cultivation areas for mussels), forewarn of the local environmental impact before damage occurs at the population, community, or ecosystem level. Such signals could be extremely helpful in mussels' farming and could provide a safeguard for the local mussel industry. The introduction of a real time "precautionary harvesting", for example, could prevent an economic loss due to mass mortality.

In the present work, using a customized magneto-electric device, the valve movements and gaping was investigated in live specimens of the Mediterranean mussel *M. galloprovincialis* exposed to variable salinity levels. In this laboratory trial, an event of drastic and abrupt reduction in salinity was used to mimic an event of unexpected and intense rain in the environment, namely "Flash Flood".

Therefore, the general aim of the present study was to estimate the resilience or inertia of valve activity of animals after a pulsing exposition to salinity, and the ability of *M. galloprovincialis* to recover. Specifically our study tested the following (null) hypotheses: a) the valve gaping behavior of mussel remained the same during the exposure of different levels of salinity; b) the valve gaping behavior of mussel remained the same after the exposure of different levels of salinity and c) the rhythm of valve movements remained unchanged during and after the exposure of different levels of salinity.

MATERIALS AND METHODS

Collection and acclimation of mussels

M. galloprovincialis specimens were collected from a mussel farm located in the Santa Gilla lagoon (Sardinia, Italy, W Mediterranean, Lat/Long 39° 13’ 48.00’’ N 9° 04’ 41.72’’ E) and transferred to the laboratory for the acclimation phase. Individuals of similar size (shell height: 65 ± 2.9 mm) were kept in experimental glass aquaria containing 9 l of filtered seawater. The protocol and procedures are full in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes.

Mussels were acclimatized over a period of 72 h under the following reference conditions: light regime of 12 h light + 12 h dark; 35 PSU (corresponding to the typical salinity of the coastal Mediterranean Sea waters), temperature 18.5 ± 0.5 °C. Oxygen was kept at saturation via constant air bubbling in the tank. The specific composition of the reference sea water is listed in Table 1. Mussels were not fed, since fasting does not affect shell movements for short-term laboratory experiments (Kramer & Foekema 2000).

Measurement of valve movements

The valve gaping of each mussel, i.e., the distance between the two valves of the shell (Vo in mm), was measured using a magneto-electric device similar to that proposed by Gnyubkin (2010). It was composed by Hall element sensors (15 × 15 × 4 mm), small magnets (10 × 6.5 × 3 mm) and a hardware system to connect sensors to the archive data recorder (Fig. 1). Nylon supports, which hold the fix Hall sensor and magnet, were glued to the valve by water resist epoxy resin (CFG©, Italy) due to its good adhesive properties on shell of mussels (Hartmann, Beggel, Auerswald & Geist 2016).

The device measured the valve gaping (recorded at interval of 5 s) in the form of the output voltage from the Hall element sensor generated by changes in the external magnetic field. Hall sensors were instrumentally calibrated at zero when valves were fully closed, and the changes in the magnetic field corresponded to changes in valves gaping. The calibration was made by the *calibration screw* which allowed to move the magnet and setup the distance of 0 mm when the valves were fully closed. The relationship between changes in the magnetic field and the opening of shell in mm was calculated and it is automatically generated by the customized software (RiFD by MC Infotronica Ltd, Italy). The RiFD allowed to routinely archive the data every 24h (CSV format) and allowed to display valve movements in real time. Since external vibration (environmental noise) can be sources of the closure of the shells, producing the closure of valves (Kramer & Foekema 2000), all trials were carried out in a soundproof laboratory at the University of Cagliari.

Experimental design

The trial simulated an event of drastic and sudden reduction (within 4 hours) of seawater salinity thus mimicking an event of unexpected and intense rain, and was aimed at investigating the resilience of exposed mussels when the initial salinity levels were recovered. The collected mussels (n = 36) were randomly assigned to four experimental levels of salinity (nine mussels per each level): salinity at 35 (reference exposure (hereinafter we will omit the salinity unit). Each experimental level of salinity considered three tanks, and each tank contained three mussels equipped with Hall sensors (Fig. 1). Reference mussels were maintained at salinity of 35 as control group for 10 days. The other mussels were exposed for 5 days to the different levels of salinity (during exposure, thereafter labeled "*During*"). The gradual exchange of salinity was obtained adding distilled water within four hours. After the 5 days of exposure, the salinity was re-established at the reference value of 35 PSU adding filtered sea water (5µm) on each experimental tank. The salinity concentration was verified instrumentally by portable conductivity meter (WTW 310, Xylem Analytics, Germany). The mussels were kept in tanks for another 5 days (after exposure, thereafter labeled "*After*"). Valves gaping, and movements were recorded simultaneously as described earlier from all mussels during the entire experiment.

Valve gaping (Vo) was recorded simultaneously during the entire experiment. Vo data for the three mussels contained in each tank were averaged prior to analysis. Filtration Activity and Transition Frequency per day were analyzed for significant differences among the treatment groups and between *"During"* and "*After*" the treatments. The Filtration Activity was measured as the fraction of time a mussel's shells were open and considered to be filtering over each day of the trial (Hartmann, Beggel, Auerswald, Stoeckle, *et al.* 2016). The Transition Frequency was the number of observations where a mussel's status changed from open to closed and vice versa for each day of the trial (Hartmann, Beggel, Auerswald, Stoeckle, *et al.* 2016). For both variables the valves were considered opened when the valve distances were higher than 0.2 mm.

Data analysis

The Kruskal–Wallis (K-W) test ($\alpha = 0.05$) was used to compare valve gaping data (Vo) from individuals kept at salinity of 5, 10, 20 and 35 PSU *During* exposure vs. individuals kept at 5, 10, 20 and 35 PSU *After* exposure.

The rhythm of valve movements (i.e. recurrence opening and closure of shells) was also analyzed to identify the occurrence of eventual oscillating or trend patterns. The Autocorrelation function (ACF) was used to identify serial dependence of gaping data *During* and *After* exposure (Zuur *et al.* 2007). ACF gives an indication of the extent of association between valve gaping data at consecutive times, Vo_t and Vo_{t+k}, where the time lag k takes the values 1, 2, 3, and so on (in minutes). Pearson's correlation coefficient was used to quantify the association of gaping data. In general, a slow moving ACF plot indicates the presence of a trend in the valve movement (for example, a continuous closing or open state), thus excluding an oscillating pattern, whereas an oscillating autocorrelation plot is evidence of a cyclical pattern of the valve activity. In this case, the patterns of cyclical data were studied using spectral analysis which uses the periodograms analysis to identify spectral densities with the highest significance of contribution to oscillations (Zuur *et al.* 2007).

Data processing and statistical analyses were performed using Brodgar 2.7.4 software (Highland Statistics Ltd, Newburgh UK).

Analysis of variance (ANOVA) was used to test for significant effects for the Filtration Activity and Transition Frequency. Prior to the analysis, Cochran's C-test ($\alpha = 0.05$) was used to check the assumption of the homogeneity of variances. Where data violated the assumption of homogeneous variances, an alpha-level adjustment to 0.01 was used to compensate for increased type I errors (Underwood 1997). Post-hoc multiple comparisons were performed using Tukey's test. STATGRAPHICS PLUS 5.1 professional edition (Statistical Graphics Corp., Rockville, MD, USA) was used for statistical analysis.

RESULTS

During the experimental period of ten days *M. galloprovincialis* specimens maintained at the reference salinity of 35 showed an average (± SD) valve gaping Vo of 1.94 ± 1.84 mm, ranging from 0.16 mm (Min) and 6.29 mm (Max) (Table 2). The K-W test showed that Vo did not vary significantly between the two experimental phases of 35 *During* vs. 35 *After* ($P = 0.55$) (Table 3).

Mussels exposed to the lowest salinity of 5 showed an average Vo of 0.73 ± 1.92 mm, ranging from 0 mm (valve completely closed) to 6.90 mm (valve almost fully open) (Table 2). Mussels remained completely closed for the first three days of the experiment (Fig. 3). During the fourth and fifth days, valves were all fully open, corresponding to the death of some of the mussels (7 out of the 9 mussels exposed to the lowest salinity died). During exposure *After*, once salinity was re-established at 35, the two surviving mussels showed Vo of 1.66 ± 1.51 mm, which was the value just below the valve gaping obtained at the reference salinity (K-W test: $P < 0.05$).

Mussels exposed to salinity of 10 kept valves fully closed for all the 5 days of exposure (Fig. 2), whereas, once the reference salinity was re-established Vo was 3.37 ± 1.54 mm, ranging between 0 (Min) and 7.74 mm (Max) (Fig. 3). In such case, the maximum value of Vo was higher than that of mussels in the reference state. The K-W test showed significant differences in Vo between the two experimental phases ($P < 0.05$).

At salinity of 20 mussels kept their valves closed during the first day (Fig. 2) and reopened the valves for the successive 4 days (Fig. 3). The maximum Vo values were higher than the valve gaping obtained at the reference salinity in both 20 *During* and 20 *After* exposure. The K-W test did not show statistical differences ($P = 0.09$).

Over the experimental period, the total filtration time of reference mussels exposed to 35 PSU was 93.22 ± 6.77% and 97.89 ± 1.51% *During* and *After*, respectively ($P > 0.05$). The Filtration Activity of mussels exposed to 20 PSU was 68.22 ± 17.99% and return to the reference values when they were exposed to 35 PSU (96.0 ± 4.0%; $P < 0.05$). At 10 PSU the mussels showed no Filtration Activity (0 ± 0%) and showed a significant decrease of the Filtration Activity when they were exposed to 35 PSU (13.89 ± 13.88%; $P < 0.05$). The same behavior occurred for mussel exposed to 5 PSU but in this case most of mussels died and the survival specimens remained closed when the salinity return to 35 PSU.

The number of transitions of each specimen exposed to 35 PSU ranged from one to seven transition per day showing a continuous flapping behavior. The Transition Frequency at 20 PSU was 4.2 ± 1.77 and 1.6 ± 0.6 *During* and *After*, respectively ($P > 0.05$). At 10 PSU the Transition Frequency was 0.40 ± 0.25 and 0.2 ± 0.2 *During* and *After* the exposure, respectively ($P < 0.05$). At 5 PSU no Transition Frequency was observed.

Since most of the specimens exposed to salinity of 5 died, the ACF analysis *During* the trial was conducted for mussels at salinity of 35 and 20 whereas all individuals at salinity of 10 had valves continuously closed for five days. The ACF for mussels at salinity 35 and 20 showed the presence of a high correlation among the first-time lag. These data indicated a trend which excluded the presence of an oscillating pattern in the valve movements (Fig. 4).

The ACF analysis for trial *After* the exposure showed a trend for specimens exposed to salinity 35 and 20, and a weak cyclic component for specimens exposed to salinity 10 (Fig. 5). Spectral analysis calculated for gaping data of mussels exposed to salinity of 10 was characterized by two peaks of spectral density: one at a low frequency of $k = 128$, representing the basal 'noise' due to the trend pattern, and a second at a frequency of $k = 300$, corresponding to a 16 h periodicity of valve flapping indicating that valves were almost fully open.

DISCUSSION

The main objective of this study was to assess the recovery or inertia of valve movements using Hall sensors on the Mediterranean mussel *M. galloprovincialis* after the exposure to different salinity levels. Here we focus primarily on the impacts of salinity stress on *M. galloprovincialis*, despite there are multiple stressors simultaneously acting upon a given organism at a particular time (Zippay & Helmuth 2012). Nevertheless, there is still a significant knowledge gap in the understanding of how each stressor contribute on the organism and the baseline for individual stress effects is far to be completed (Crain *et al.* 2008). Although there are several examples on how environmental factors influenced the valve gape behavior on mussels (Kramer 2009; Burnett *et al.* 2013; Beggel & Geist 2015; Lummer *et al.* 2016; Redmond *et al.*

2017), magneto-electric devices have been applied to the Mediterranean mussel *M. galloprovincialis* only once (Gnyubkin 2010).

The results presented here showed that under reference conditions of salinity of 35 (corresponding to the typical salinity of the coastal Mediterranean Sea waters) and fasting, the general patterns of *M. galloprovincialis* valve movements revealed a continuously open state with sporadic spikes indicating a closing motion. In past studies (Kramer & Foekema 2000), shell open behavior with sporadic closing and re-opening of shells in the range of 70 - 80% of the time, were usually associated for food and oxygen intake and explained as normal behavior in valve movement of mussels.

The drastic reduction of salinity tested, which mimicked an event of sudden and intense rain, had a significant effect on valve movements and on the survival of mussels. Indeed, the exposure to a salinity of 5, a concentration that is well below the optimal tolerance range of *M. galloprovincialis*, lead to the highest mortality of individuals. In detail, mussels remained completely closed for the first three days of the experiment and died during the fourth and fifth days of the trial showing continuous gaping (no further movement and 100% opening of valves). This result corroborates a previous investigation which demonstrated that extreme osmotic stress at low salinity enhanced mortality in *M. galloprovincialis* after 14 days of progressive salinity acclimation (Hamer et al. 2008). In particular, the closing of mussel shells is considered indicative of escape or defense behavior under stress conditions (de Zwart *et al.* 1995; Borcherding 2006; Gnyubkin 2010).

At salinity of 10 all mussels remained closed. When the environmental conditions returned to pristine, they showed a reduction on the Transition Frequency and Filtration Activity confirming that *M. galloprovincialis* showed a high resistance to the salinity tested (Van Erkom Schurink, C Griffiths 1993; Branch & Nina Steffani 2004) but with a negative effect on the valve movements. At salinity of 20 mussels reacted with a small reduced gaping but showed the capacity to regain valve gaping similar to the behavior at the reference state. In such case mussels revealed an “indifferent” behavior in respect to salinity tested. This was also in accordance with the results obtained for a group of mussels acclimated to salinity of 18.5 (Hamer *et al.* 2008).

The extreme variability of salinity tested in our trials does not represent the normal environmental conditions in intertidal zones and estuaries areas of the Mediterranean.

Nevertheless, in recent years unprecedented mussel's mass mortality occurred in many intertidal and estuaries areas of the Mediterranean and north Atlantic as consequence of abrupt drop of salinity caused by extreme run-off after heavy rain events, namely "Flash Flood" (Bechemin *et al.* 2015; Benabdelmouna & Ledu 2016; Polsenaere *et al.* 2017). Transitional waters and the associated biodiversity are susceptible to constantly low salinities, frequency and amplitude of salinity changes, as well as the changing rate of salinity. Each of these osmotic variables influences behavioral responses of shellfish (e.g. shell valve closure), as well as filtration activity, growth rate, early development and survival rate (Bøhle 1972; Qiu *et al.* 2002). These salinity-related physiological stresses on shellfish are destined to increase in the future as consequence of extreme climatic events which will affect both the Mediterranean Europe and North Atlantic. For example, one of the most supported climate change scenario for the Baltic Sea predicts that an increased riverine input of freshwater will result in a further reduction in salinity in intertidal zones and estuaries areas (Johannesson *et al.* 2011). According to these authors this scenario will favor establishment and spread of freshwater species in these habitats and the progressive disappearance of stenohaline sessile species, including shellfish. Moreover, the increasing of coastal flooding will be the main vector of fine sediments delivery. This is considered another important stressors of aquatic organisms either through sedimentation and clogging of the stream bed, through increased turbidity, or as a source of adsorbed chemicals such as nutrients or contaminants affecting water quality (Lummer *et al.* 2016).

Such climatic trends certainly will affect the suitability of geographical locations for aquaculture facilities and particularly the European mussel industry with strong consequences in the economy of several countries where mussels represents a high-value market (Polsenaere *et al.* 2017; Eumofa 2019).

Our experimental study simulated three scenarios of unexpected and intense Flash Flood events which lasted for five days. These scenarios were not so far from the significant reductions in PSU that may occur in the environment. In some coastal lagoons and aquatic transitional environments of the Mediterranean ecoregion these low salinities can last for weeks, especially in the first 50 cm of water from the surface. This was observed recently in some lagoons of Sardinia after Flash flood events (Authors personal observation). The valve gape behavior observed during our trails showed that

M. galloprovincialis is not capable to recover when subjected to a pulse disturbance generated by salinity of 10, whereas at salinity of 5 was observed a high mortality. In contrast, salinity of 20 did not trigger a significant behavioral response during the exposure period. The quick response to the selected stressors of mussels using the Hall sensors device, would be helpful as “early warning signals” in mussel farming industry. The positioning of the magneto electric device in the areas suitable for mussels’ farming would allow to forewarn local deterioration of water quality or local impacts and to adopt real time safeguard approaches. For example, a precautionary “early harvesting” or the moving of the mussel’s cultivation off-the-coast or offshore could be the best practice to adopt. The last two options are currently considered promising industry in mussel aquaculture to reduce the risk due to the changing environment (Mizuta & Wikfors 2019).

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Table 1 Summary of sea water reference chemistry parameters for valve behavior of *M. galloprocialis*

Parameter	Concetration (ppm)
Nitrate (NO3-)	21.352
Nitrite (NO2-)	0.007
Silicate (SiO2)	0.378
Total Phosphate	0.010

Table 2 Descriptive statistics for valve gaping of *M. galloprovincialis* for *During* (d) considering three seawater salinity (5, 10 and 20 PSU) and the reference state (35 PSU), and *After* (a) when the reference state was re-established in each treatment (Vo: valve gaping; Es: standard error; SD: standard deviance; V: variance; Min: minimum gaping; Max: maximum gaping; d: trial *During*; a: trial *After*).

Vo (mm)	5_d	5_a	10_d	10_a	20_d	20_a	35_d	35_a
Mean	0.73	1.66	0	3.37	1.63	1.89	1.93	1.95
Es	0.05	0.05	0	0.05	0.03	0.06	0.03	0.03
SD	1.92	1.51	0	1.54	1.35	1.91	1.37	1.34
V	3.69	2.29	0	2.38	1.84	3.64	1.88	1.79
Min	0	0	0	0	0	0.02	0.16	0.16
Max	6.90	5.63	0	7.74	7.60	8.11	6.29	6.29

Table 3 Results of the Kruskal-Wallis test comparing valve gaping (Vo) data *During* vs. *After* treatment, i.e. when the reference state of salinity (35 PSU) was re-established (d: trial *During*; a: trial *After*)

	5_d	5_a	10_d	10_a	20_d	20_a	35_d	35_a
Avg. Rank	703.3	1097.6	451.5	1349.5	921	879.9	907.8	893.1
P	< 0.05		< 0.05		0.09		0.55	

FIGURE LEGENDS

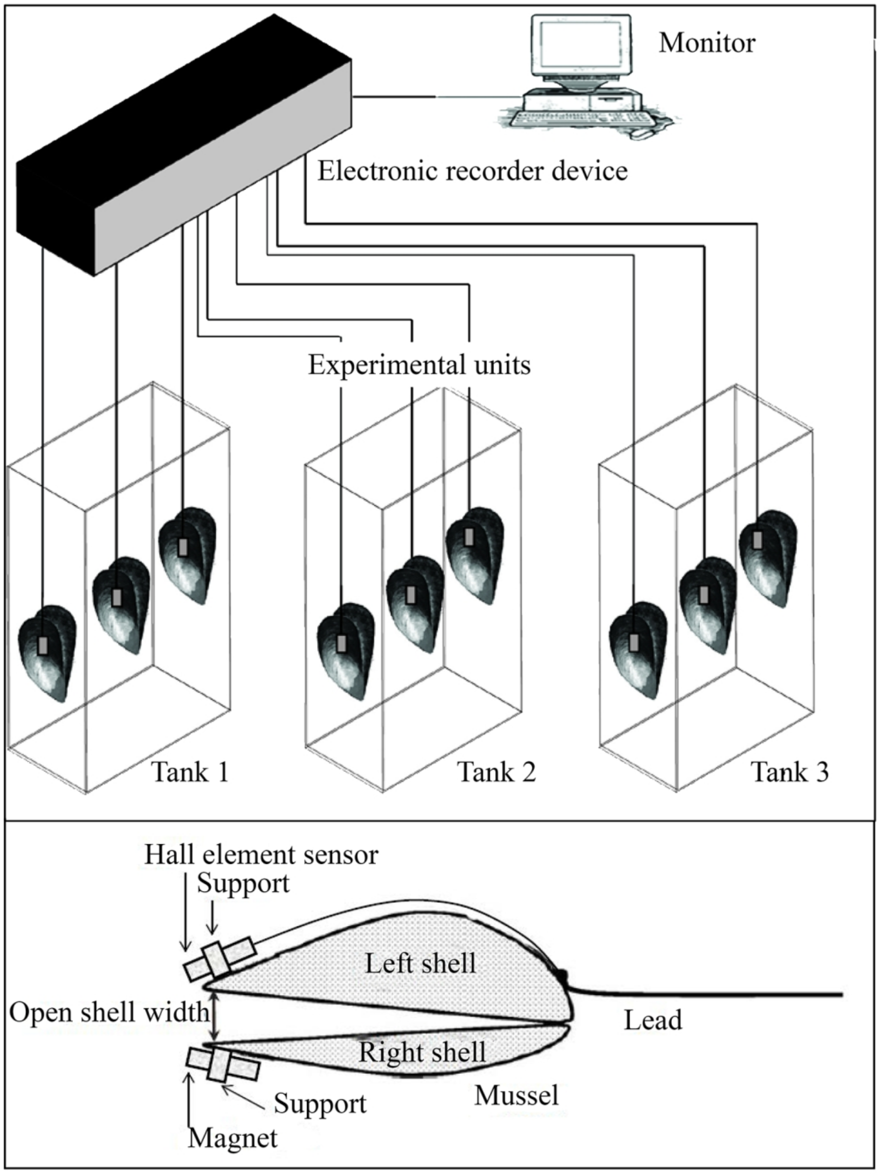
Figure 1 Scheme of the valvometer device utilized for the experiment (above), and detail of the connection of the Hall's sensor–magnet to mussel valves (below).

Figure 2 Box plots of the valve gaping (Vo) for three classes of salinity (5, 10 and 20 PSU) and the reference salinity (35 PSU) during recordings of day 1-5.

Figure 3 Box plots of the valve gaping (Vo) for three class of salinity (5, 10 and 20 PSU and the reference salinity (35 PSU) during recordings of day 6-10 when the reference state was re-established. Vo at salinity of 5 is referred two survived mussels.

Figure 4 Autocorrelation function (ACF) for valve gaping (Vo) recordings at salinity 20 and 35 PSU considering the *During* exposure.

Figure 5 Autocorrelation function (ACF) for valve gaping (Vo) recordings at salinity 10, 20 and 35 PSU considering the *After* exposure.



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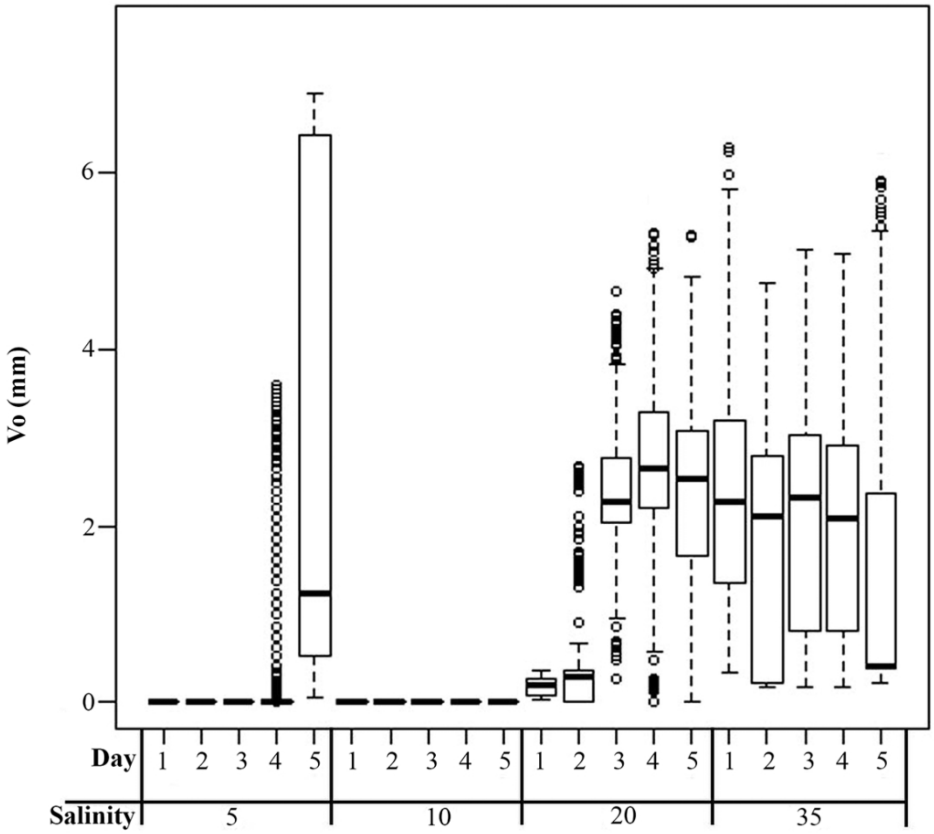

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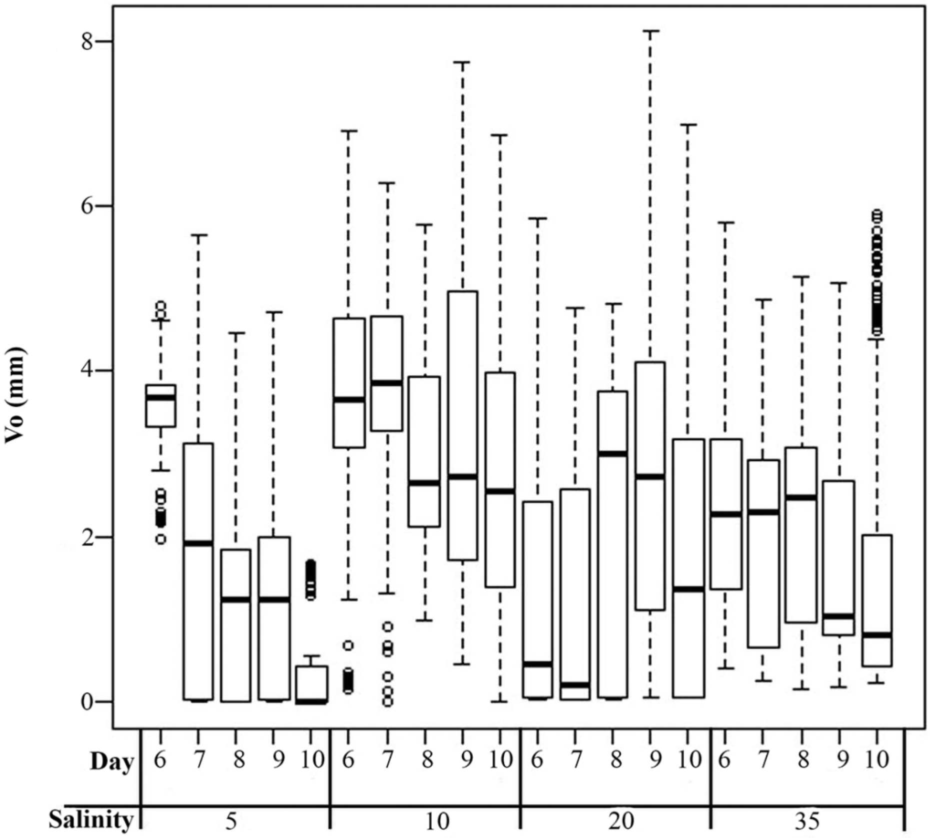


Figure 3 Box plots of the valve gaping (Vo) for three class of salinity (5, 10 and 20 PSU and the reference salinity (35 PSU) during recordings of day 6-10 when the reference state was re-established. Vo at salinity of 5 is referred two survived mussels.

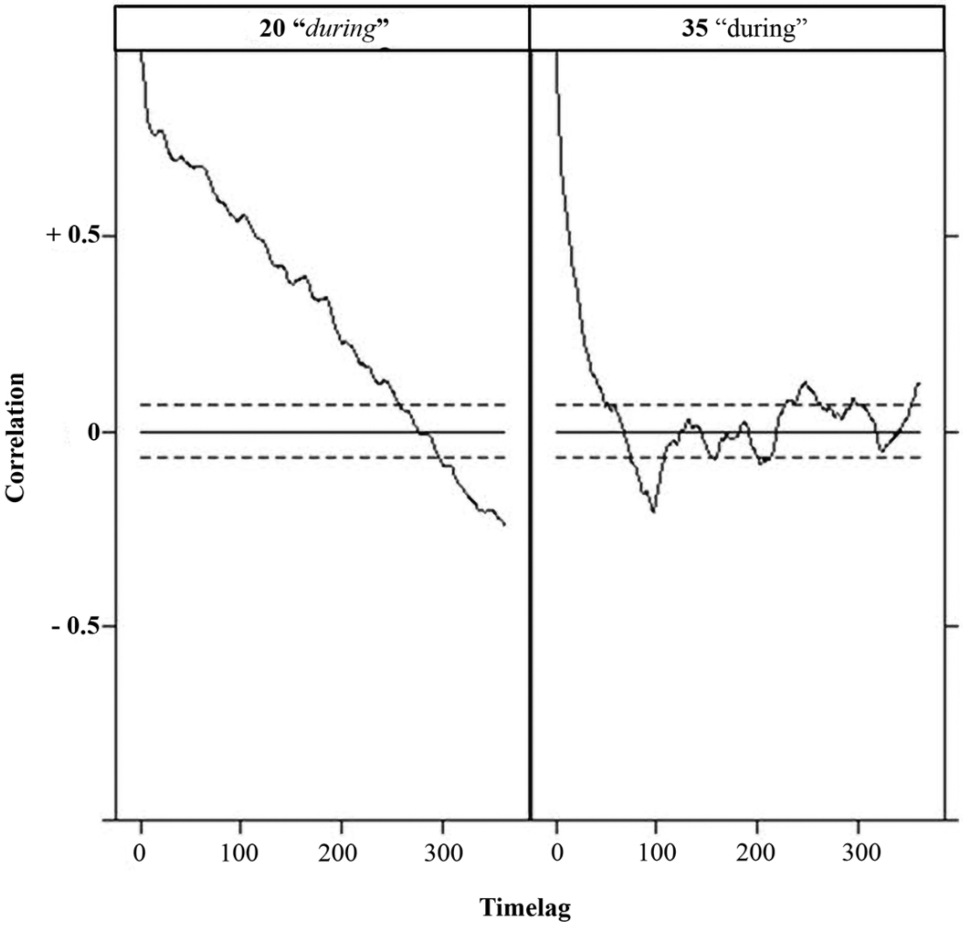


Figure 4 Autocorrelation function (ACF) for valve gaping (Vo) recordings at salinity 20 and 35 PSU considering the During exposure.

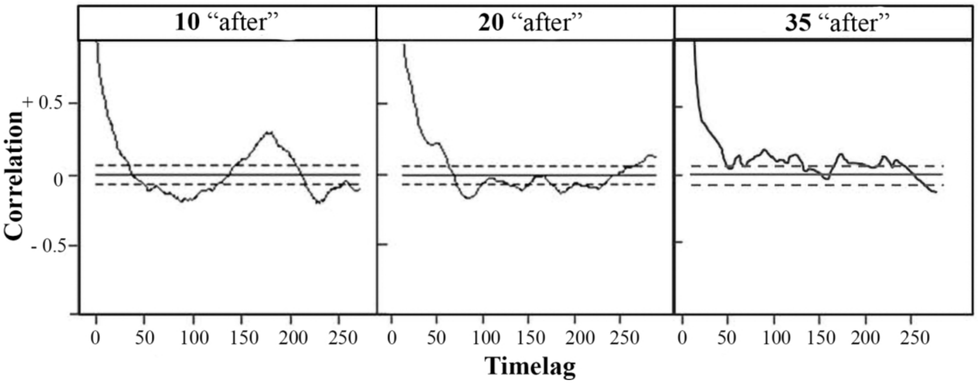
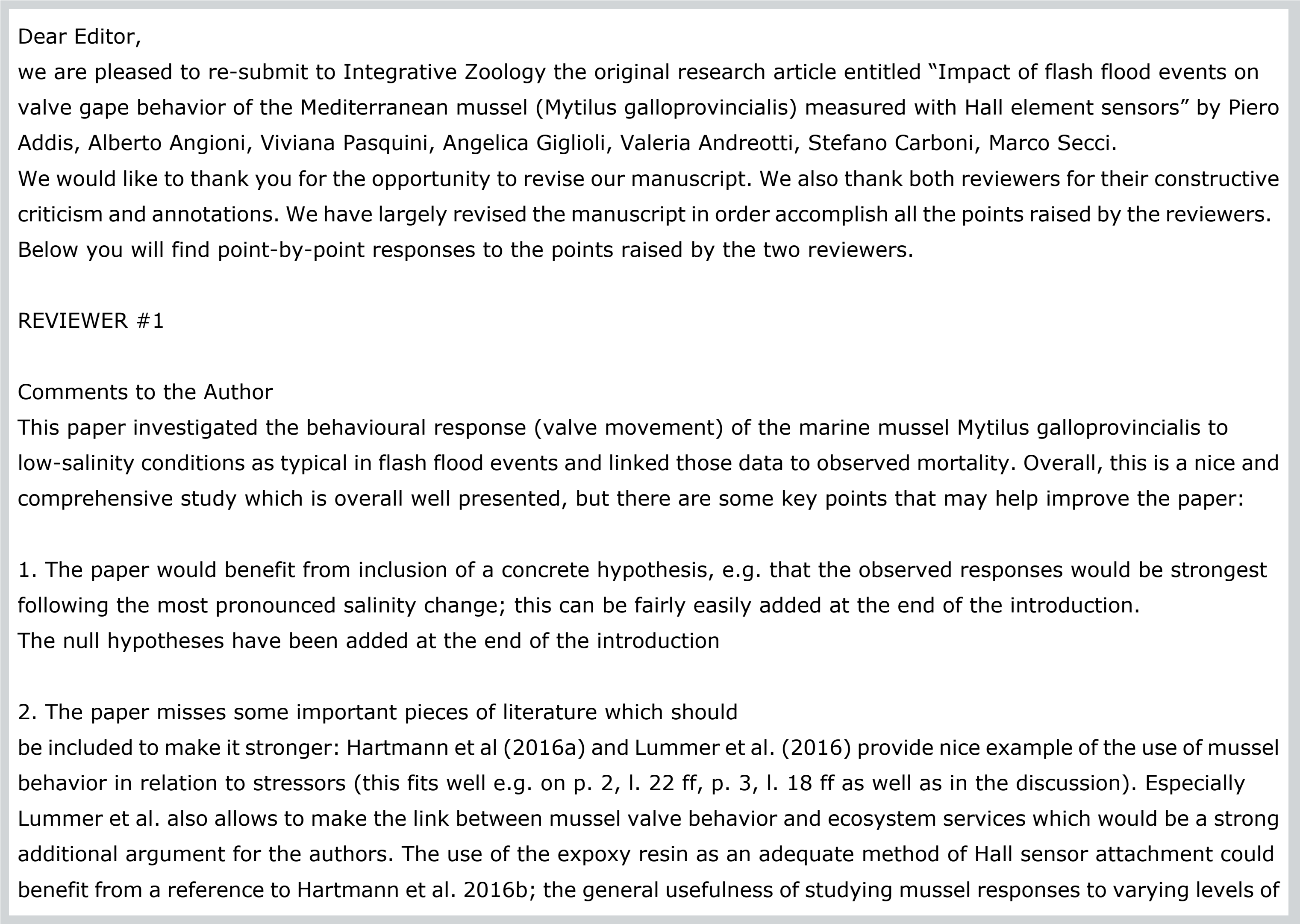


Figure 5 Autocorrelation function (ACF) for valve gaping (Vo) recordings at salinity 10, 20 and 35 PSU considering the After exposure.



Dear Editor,

we are pleased to re-submit to Integrative Zoology the original research article entitled “Impact of flash flood events on valve gape behavior of the Mediterranean mussel (Mytilus galloprovincialis) measured with Hall element sensors” by Piero Addis, Alberto Angioni, Viviana Pasquini, Angelica Giglioli, Valeria Andreotti, Stefano Carboni, Marco Secci.

We would like to thank you for the opportunity to revise our manuscript. We also thank both reviewers for their constructive criticism and annotations. We have largely revised the manuscript in order accomplish all the points raised by the reviewers. Below you will find point-by-point responses to the points raised by the two reviewers.

REVIEWER #1

Comments to the Author

This paper investigated the behavioural response (valve movement) of the marine mussel Mytilus galloprovincialis to low-salinity conditions as typical in flash flood events and linked those data to observed mortality. Overall, this is a nice and comprehensive study which is overall well presented, but there are some key points that may help improve the paper:

1. The paper would benefit from inclusion of a concrete hypothesis, e.g. that the observed responses would be strongest following the most pronounced salinity change; this can be fairly easily added at the end of the introduction.

The null hypotheses have been added at the end of the introduction

2. The paper misses some important pieces of literature which should be included to make it stronger: Hartmann et al (2016a) and Lummer et al. (2016) provide nice example of the use of mussel behavior in relation to stressors (this fits well e.g. on p. 2, l. 22 ff, p. 3, l. 18 ff as well as in the discussion). Especially Lummer et al. also allows to make the link between mussel valve behavior and ecosystem services which would be a strong additional argument for the authors. The use of the expoxy resin as an adequate method of Hall sensor attachment could benefit from a reference to Hartmann et al. 2016b; the general usefulness of studying mussel responses to varying levels of

salinity would be well-supported by citing Beggel & Geist (2015), particularly since this study considered the exact inverse situation of freshwater mussels being exposed to high-salinity stressors
These relevant literatures have been added in the text.

3. Some more information on methodological details and appropriate testing conditions is needed (as specified below when referring to concrete pages in the text).
The information required has been added as you can see in the specific information below.

4. The analyses and data presentation (including Figures) may benefit from also considering analyses of activity patterns and transition frequencies (see Hartmann et al. 2016a)
As you suggested, we improve the manuscript with the activity pattern and the transition frequency.

. Figures: Please correct typos (Figure 1: needs to read “open shell width (with two “l”); Figure legend 2: “classes” instead of “class” and consider either explaining the outliers or including them as datapoints (same for Fig 3); generally it may be more useful to draw response patterns graphs instead of box blots.
The figure and the legend have been modified. We use the Box–Whisker plots since they well represent several gape behavior of mussels as also reported by Hartmann et al. (2016a).

Specific information:

Page 4 Line 34: Please provide more details on how distance was calculated Line 43: Do 5 s provide enough resolution to identify the small spikes?
The information required has been added in the text. The resolution of 5s second was a balance to identify the small pikes and reduce the space of files to store the data on valve gaping along the entire trial.

Line 46: “Hall sensors were instrumentally calibrated at zero when valves were fully closed‟ – please explain Line 55: Information on alarm criteria are missing

The calibration method was added in the MM section. For alarm we mean the closure of valves for several times even at standard conditions.

Page 5 “Experimental design”: The description here is lacking substantial information of the test conditions and quality control. For exposure experiments using mussels the ASTM Guideline “E2455-05. Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels. DOI:10.1520/E2455-06” gives a good orientation for reporting criteria.

- How were the mussels transferred to the exposure aquarium? It seems they were moved manually from one aquarium to the other, wouldn´t a gradual exchange of the test medium have been more appropriate?

We made a gradual exchange and we improve the manuscript on how it was done.

- Information on water quality parameters or the water matrix are generally missing.

The specific composition of the reference sea water is listed in a new table (Table 1).

- How were salinity levels maintained and measured?

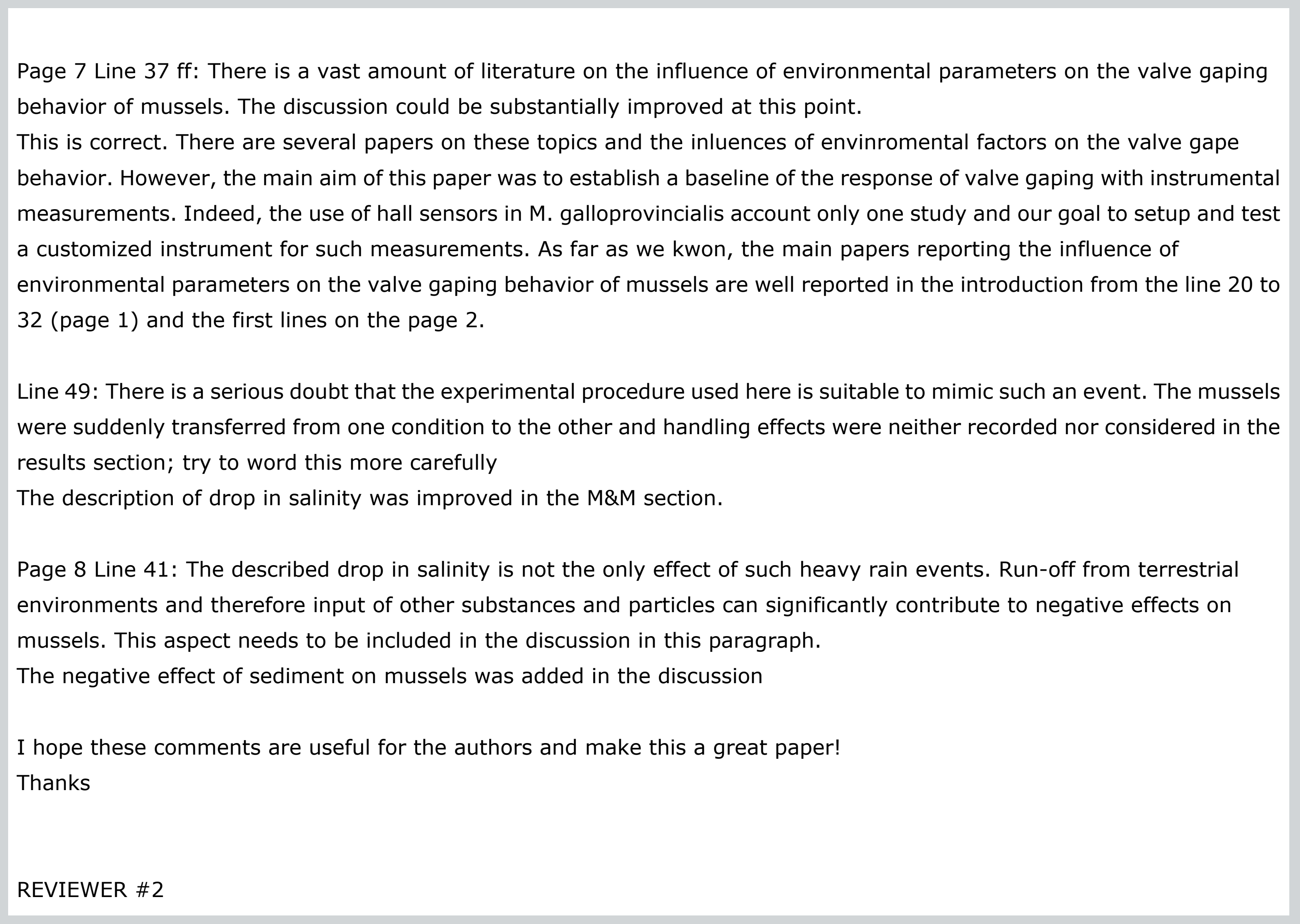
The salinity was verified instrumentally by portable conductivity meter. We improve the text on this regard.

Line 22: How was salinity “re-established”?

We explained in the text how it was re-established.

Page 6 I would suggest to not only report gaping distances but also include an analysis about activity patterns and transition frequencies, see Hartmann et al. (2016a) for further details.

The manuscript was improved with the activity pattern and transition frequency adding the findings in the results and their relative methods.



Page 7 Line 37 ff: There is a vast amount of literature on the influence of environmental parameters on the valve gaping behavior of mussels. The discussion could be substantially improved at this point.

This is correct. There are several papers on these topics and the inluences of envinromental factors on the valve gape behavior. However, the main aim of this paper was to establish a baseline of the response of valve gaping with instrumental measurements. Indeed, the use of hall sensors in M. galloprovincialis account only one study and our goal to setup and test a customized instrument for such measurements. As far as we kwon, the main papers reporting the influence of environmental parameters on the valve gaping behavior of mussels are well reported in the introduction from the line 20 to 32 (page 1) and the first lines on the page 2.

Line 49: There is a serious doubt that the experimental procedure used here is suitable to mimic such an event. The mussels were suddenly transferred from one condition to the other and handling effects were neither recorded nor considered in the results section; try to word this more carefully

The description of drop in salinity was improved in the M&M section.

Page 8 Line 41: The described drop in salinity is not the only effect of such heavy rain events. Run-off from terrestrial environments and therefore input of other substances and particles can significantly contribute to negative effects on mussels. This aspect needs to be included in the discussion in this paragraph.

The negative effect of sediment on mussels was added in the discussion

I hope these comments are useful for the authors and make this a great paper!

Thanks

REVIEWER #2

General comments

The study here describes an increasingly important monitoring tool for detecting perturbations in the environment i.e. shellfish gape behavior. Whilst adequately done, the study is a little one dimensional in that it only considers valve closure. There was an opportunity to test effects on osmotic effects on in order to test what is causing mortality and/or monitor what triggers valve closure.

Abstract:

Generally fine and clear. Structure of some sentences need to be improved i.e. include PSU after each mention of experimental salinity tested

We included the PSU for each salinity level.

L25-29: "Data recordings of valve gaping were analyzed by Kruskal–Wallis test while the rhythm of valve movements was studied using Autocorrelation function and Spectral analysis." This detail not needed here as it adds nothing.

The sentence has been deleted.

L41: change to "….no define rhythms of valve movements was ….

We changed the sentence.

Introduction

P2 L15: climate change

It was changed.

L39: gape

It was changed

L44: recurrence of
It was changed

P3
L29-34: this sentence doesn’t make sense to me. Please rewrite.
We rewrite the sentence

L42-43: more justification for the studying of flash floods is needed. Is this a natural perturbation frequently experienced by mussels in the wild, if so, how frequently? Is this expected to change in the future i.e. might precipitation patterns be predicted to change under climate change? Are more intense rain events predicted? Some more justification here is needed.
We improve this part with some examples of flash floods.

Materials and methods
Replicates are awfully low for a relatively simple experiment.
Probably you are wright, but our custom device can manage simultaneously this low number of mussels.

P4
L15: change to nine
It was changed

P5:
L15: My preference is to keep the salinity unit for clarity
The PSU unit is now reported in the whole manuscript

L21: do you have any data on how long reduced salinity events persist in the environment of the mussel? From my experience significant reductions in PSU from intense rainfall are transitory and usually pass within 3 days or less. Of course, that may reflect local conditions where I live, so is there any environmental data on Sardinia to justify your experimental protocol?
In Sardinia, we monitored some coastal lagoon where mussel farming represents the main aquaculture activity. In some of these locations, as consequence of flash flood events, the low salinity can last for months especially in the first 50 cm of water from the surface.

L36; Why Kruskal-Wallis? Did your data not meet the assumptions of ANOVA or other? What are you testing here gape width in mm, or frequency of valve closure?
Since the data did not meet the assumptions of ANOVA, we used a non-parametric test.

To my mind comparing just gape width is a little pointless as it will always be highly variable i.e. range from 0 – 7 mm, thus unlikely to produce a statistically useful number. A better approach is to use a combination shell integral value i.e. duration open + gape width, as laid out in Powell JA, Ragg NLC, Dunphy BJ (2017) Phenotypic biomarkers in selectively-bred families of the Greenshell™ mussel (Perna canaliculus): Anaerobic enzyme and shell gape behaviour as biomarkers of prolonged emersion tolerance. Aquaculture 479: 601-608 doi http://dx.doi.org/10.1016/j.aquaculture.2017.06.038. This tells us how wide and for how long mussel valves are open, and is a more informative behavioural response.
As you and the other reviewer suggested, we improved the manuscript with more behavioral responses such as the activity pattern (how long mussel valves were open) and the transition frequency analysis (which is the frequency of opening/closure of valves per day). The ANOVA test has been used for such analysis.

P6
Results
L15-21: echoes what I mention above, a bit of a pointless comparison in my mind, it was always going to be highly variable

thus result in non-significance.
You are right and as you see in the above comment, we improve the manuscript with new results.

L32-35: and this where your low replicate numbers are a problem, comparing two mussels isn't much of a powerful test and really, limits the conclusions you can draw.
Thank you for your comments and observations. Currently the device can host only 9 sensors. In order to improve the number of replicates we are implementing a new device will host up to 16 sensors. In this way we plan to improve the statistical significance in the data recordings.

Discussion
P7
L53: would the salinity ever drop this far? It would require a huge volume of fresh water. Nonetheless, it fascinating to me that these mussels die after such a small period of time, indicating that valve closure does not completely seal them off from the environment.
We made a gradual exchange and we improve the manuscript on how it was done.

P8
L34-56: This is needed in Introduction to justify the study. Are these cyclones predicted to increase? I'm assuming these are times when the water temperatures are very warm, so mussels are low in physiological condition in the wild, coupled with potential effects of summer mortality.
We move this part in the introduction