



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

Journal:	<i>Integrative Zoology</i>
Manuscript ID	INZ-2020-03-080.R1
Manuscript Type:	Original Article
Keywords:	Mussels, <i>Mytilus galloprovincialis</i> , Valve activity, Hall sensor, Salinity
Specialty:	Marine Biology, Ecology, Behavior

SCHOLARONE™
Manuscripts

This is the peer reviewed version of the following article: Addis, P, Angioni, A, Pasquini, V et al. (2021). Flash flood simulation and valve behavior of *Mytilus galloprovincialis* measured with Hall sensors. *Integrative Zoology* 16, 138-148, which has been published in final form at <https://doi.org/10.1111/1749-4877.12471>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for self-archiving.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

4 **Abstract**

5 Mussels close their shell as a protective strategy and the quantification of this
6 behavioral marker may represent an alarm signal when they are exposed to
7 environmental stressors. In the present study, we investigated the ability of the
8 Mediterranean mussel *Mytilus galloprovincialis* to recover and then the resilience or
9 inertia of valve activity after a pulsing exposition to diverse levels of salinity (5, 10, 20
10 and 35 PSU as reference value). The trial simulated an event of drastic and sudden
11 reduction of seawater salinity thus mimicking an event of Flash Flood from intense rain.
12 Valve gaping and movements were measured in continuous cycle for ten days using a
13 customized magneto-electric device which uses Hall sensors. Results showed that under
14 normal conditions of salinity (35 PSU) the general pattern of valve movements was a
15 continuously open state with sporadic spikes indicating a closing motion. At salinity of
16 5 PSU mussels reacted by closing their valves, leading to a 77% mortality on the fourth
17 day. At salinity of 10 PSU animals were observed with closed valves for the entire
18 duration of the exposure and no mortality occurred, they showed a significant reduction
19 in the valve activity once the reference value of salinity was re-established. In contrast,
20 salinity of 20 PSU did not trigger a significant behavioral response. Interestingly, there
21 no define rhythms of valve movements were recorded during salinity challenges.

23 **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.*

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 (5 μ m) 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 (V_o) 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, V_{o_t} and $V_{o_{t+k}}$, 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 (\pm 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 $93.22 \pm 6.77\%$ and $97.89 \pm 1.51\%$ *During* and *After*, respectively ($P > 0.05$). The Filtration Activity of mussels exposed to 20 PSU was $68.22 \pm 17.99\%$ and return to the reference values when they were exposed to 35 PSU ($96.0 \pm 4.0\%$; $P < 0.05$). At 10 PSU the mussels showed no Filtration Activity ($0 \pm 0\%$) and showed a significant decrease of the Filtration Activity when they were exposed to 35 PSU ($13.89 \pm 13.88\%$; $P < 0.05$). The same behavior occurred for mussel exposed to 5 PSU but in

1 this case most of mussels died and the survival specimens remained closed when the
2 salinity return to 35 PSU.

3 The number of transitions of each specimen exposed to 35 PSU ranged from one to
4 seven transition per day showing a continuous flapping behavior. The Transition
5 Frequency at 20 PSU was 4.2 ± 1.77 and 1.6 ± 0.6 *During* and *After*, respectively ($P >$
6 0.05). At 10 PSU the Transition Frequency was 0.40 ± 0.25 and 0.2 ± 0.2 *During* and
7 *After* the exposure, respectively ($P < 0.05$). At 5 PSU no Transition Frequency was
8 observed.

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

15 The ACF analysis for trial *After* the exposure showed a trend for specimens exposed to
16 salinity 35 and 20, and a weak cyclic component for specimens exposed to salinity 10
17 (Fig. 5). Spectral analysis calculated for gaping data of mussels exposed to salinity of
18 10 was characterized by two peaks of spectral density: one at a low frequency of $k =$
19 128, representing the basal 'noise' due to the trend pattern, and a second at a frequency
20 of $k = 300$, corresponding to a 16 h periodicity of valve flapping indicating that valves
21 were almost fully open.

22 DISCUSSION

23 The main objective of this study was to assess the recovery or inertia of valve
24 movements using Hall sensors on the Mediterranean mussel *M. galloprovincialis* after
25 the exposure to different salinity levels. Here we focus primarily on the impacts of
26 salinity stress on *M. galloprovincialis*, despite there are multiple stressors
27 simultaneously acting upon a given organism at a particular time (Zippay & Helmuth
28 2012). Nevertheless, there is still a significant knowledge gap in the understanding of
29 how each stressor contribute on the organism and the baseline for individual stress
30 effects is far to be completed (Crain *et al.* 2008). Although there are several examples
31 on how environmental factors influenced the valve gape behavior on mussels (Kramer
32 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; Borchertding 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.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 Nevertheless, in recent years unprecedented mussel’s mass mortality occurred in many
2 intertidal and estuaries areas of the Mediterranean and north Atlantic as consequence of
3 abrupt drop of salinity caused by extreme run-off after heavy rain events, namely “Flash
4 Flood” (Bechemin *et al.* 2015; Benabdelmouna & Ledu 2016; Polsenaere *et al.* 2017).
5 Transitional waters and the associated biodiversity are susceptible to constantly low
6 salinities, frequency and amplitude of salinity changes, as well as the changing rate of
7 salinity. Each of these osmotic variables influences behavioral responses of shellfish
8 (e.g. shell valve closure), as well as filtration activity, growth rate, early development
9 and survival rate (Bøhle 1972; Qiu *et al.* 2002). These salinity-related physiological
10 stresses on shellfish are destined to increase in the future as consequence of extreme
11 climatic events which will affect both the Mediterranean Europe and North Atlantic. For
12 example, one of the most supported climate change scenario for the Baltic Sea predicts
13 that an increased riverine input of freshwater will result in a further reduction in salinity
14 in intertidal zones and estuaries areas (Johannesson *et al.* 2011). According to these
15 authors this scenario will favor establishment and spread of freshwater species in these
16 habitats and the progressive disappearance of stenohaline sessile species, including
17 shellfish. Moreover, the increasing of coastal flooding will be the main vector of fine
18 sediments delivery. This is considered another important stressors of aquatic organisms
19 either through sedimentation and clogging of the stream bed, through increased
20 turbidity, or as a source of adsorbed chemicals such as nutrients or contaminants
21 affecting water quality (Lummer *et al.* 2016).
22 Such climatic trends certainly will affect the suitability of geographical locations for
23 aquaculture facilities and particularly the European mussel industry with strong
24 consequences in the economy of several countries where mussels represents a high-
25 value market (Polsenaere *et al.* 2017; Eumofa 2019).
26 Our experimental study simulated three scenarios of unexpected and intense Flash
27 Flood events which lasted for five days. These scenarios were not so far from the
28 significant reductions in PSU that may occur in the environment. In some coastal
29 lagoons and aquatic transitional environments of the Mediterranean ecoregion these low
30 salinities can last for weeks, especially in the first 50 cm of water from the surface. This
31 was observed recently in some lagoons of Sardinia after Flash flood events (Authors
32 personal observation). The valve gape behavior observed during our trails showed that

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

1 **REFERENCES**

2 Ameyaw-Akumfi C, Naylor E (1987). Temporal patterns of shell-gape in *Mytilus*
3 *edulis*. *Marine Biology* **95**, 237–242.

4 Bae MJ, Park YS (2014). Biological early warning system based on the responses of
5 aquatic organisms to disturbances: A review. *Science of the Total Environment* **466–**
6 **467**, 635–649.

7 Baldwin IG, Kramer KJM (1994). Biological early warning systems (BEWS). In:
8 Kramer KJM, ed. *Biomonitoring of Coastal Waters and Estuaries*. CRC Press, Boca
9 Raton, pp. 1–23.

10 Bechemin C, Soletchnik P, Polsenaere P, Le Moine O, Pernet F, Protat M et al. (2015).
11 Episodes de mortalité massive de moules bleues observés en 2014 dans les Pertuis
12 charentais. *Bulletin Epidémiologie, Santé animale et alimentation* **67**, 6–9.

13 Beggel S, Geist J (2015). Acute effects of salinity exposure on glochidia viability and
14 host infection of the freshwater mussel *Anodonta anatina* (Linnaeus, 1758). *Science of*
15 *the Total Environment* **502**, 659–665.

16 Benabdelmouna A, Ledu C (2016). The mass mortality of blue mussels (*Mytilus spp.*)
17 from the Atlantic coast of France is associated with heavy genomic abnormalities as
18 evidenced by flow cytometry. *Journal of the Invertebrate Pathology* **138**, 30–38.

19 Bøhle B (1972). Effects of adaptation to reduced salinity on filtration activity and
20 growth of mussels (*Mytilus edulis* L.). *Journal of Experimental Marine Biology and*
21 *Ecology* **10**, 41–47.

22 Borcherding J (2006). Ten years of practical experience with the Dreissena-Monitor, a
23 biological early warning system for continuous water quality monitoring.
24 *Hydrobiologia* **556**, 417–426.

25 Branch GM, Nina Steffani C (2004). Can we predict the effects of alien species? A
26 case-history of the invasion of South Africa by *Mytilus galloprovincialis* (Lamarck).
27 *Journal of Experimental Marine Biology and Ecology* **300**, 189–215.

28 Burnett NP, Seabra R, De Pirro M, Wethey DS, Woodin SA, Helmuth B et al. (2013).
29 An improved noninvasive method for measuring heartbeat of intertidal animals.
30 *Limnology and Oceanography: Methods* **11**, 91–100.

31 Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C, Viarengo A (2000). The
32 use of biomarkers to assess the impact of pollution in coastal environments of the

- 1 Iberian Peninsula: A practical approach. *Science of the Total Environment* **247**, 295–
- 2 311.
- 3 Caza F, Cledon M, St-Pierre Y (2016). Biomonitoring climate change and pollution in
- 4 marine ecosystems: A review on *Aulacomya ater*. *Journal of Marine Biology* **2016**.
- 5 Crain CM, Kroeker K, Halpern BS (2008). Interactive and cumulative effects of
- 6 multiple human stressors in marine systems. *Ecology Letters* **11**, 1304–1315.
- 7 Dharmaraj S (1983). Oxygen consumption in pearl oyster *Pinctada fucata* (Gould) and
- 8 *Pinctada sugillata* (Reeve). In: Silas BG, ed. *Proceedings of Symposium Coastal*
- 9 *Aquaculture Part 2: Molluscan Culture*. Marine Biological Association of India,
- 10 Cochin, pp. 627–632.
- 11 Dowd WW, Somero GN (2013). Behavior and survival of *Mytilus* congeners following
- 12 episodes of elevated body temperature in air and seawater. *Journal of Experimental*
- 13 *Biology* **216**, 502–514.
- 14 Van Erkom Schurink, C Griffiths CL (1993). Factors affecting relative rates of growth
- 15 in four South African mussel species. *Aquaculture* **109**, 257–273.
- 16 Eumofa (2019). *Case Study Fresh Mussel in the Eu in the Supply Chain*. European
- 17 Market Observatory for Fisheries and Aquaculture Products, Belgium, Bruxelles.
- 18 Fujii T (1977). Measurement of periodic open and shut shell movement of bivalves by
- 19 the strain-gauge method. *Bulletin. of the Japanese Society of Scientific Fisheries* **47**,
- 20 901.
- 21 Fujii T, Toda S (1991). Open and close shell-movement of the mussel, *Mytilus edulis* L.
- 22 under natural conditions. *National Research Institute of Aquaculture* **20**, 33–40.
- 23 Gainey LF, Shumway SE (1988). A compendium of the responses of bivalve mollusks
- 24 to toxic dinoflagellates. *Journal of Shellfish Research* **7**, 623–628.
- 25 Gnyubkin VF (2010). The circadian rhythms of valve movements in the mussel *Mytilus*
- 26 *galloprovincialis*. *Russian Journal of Marine Biology* **36**, 419–428.
- 27 Goldberg ED (1975). The mussel watch - A first step in global marine monitoring.
- 28 *Marine Pollution Bulletin* **6**, 111.
- 29 Goldberg ED (1978). The Mussel Watch. *Environromental Conservation* **5**, 101–125.
- 30 Halldórsson HP, De Pirro M, Romano C, Svavarsson J, Sarà G (2008). Immediate
- 31 biomarker responses to benzopyrene in polluted and unpolluted populations of the blue
- 32 mussel (*Mytilus edulis* L.) at high-latitudes. *Environment International* **34**, 483–489.

- 1 Hamer B, Jakšić Ž, Pavičić-Hamer D, Perić L, Medaković D, Ivanković D et al. (2008).
2 Effect of hypoosmotic stress by low salinity acclimation of Mediterranean mussels
3 *Mytilus galloprovincialis* on biological parameters used for pollution assessment.
4 *Aquatic Toxicology* **89**, 137–151.
- 5 Hartmann JT, Beggel S, Auerswald K, Geist J (2016). Determination of the most
6 suitable adhesive for tagging freshwater mussels and its use in an experimental study of
7 filtration behaviour and biological rhythm. *Journal of Molluscan Studies* **82**, 415–421.
- 8 Hartmann JT, Beggel S, Auerswald K, Stoeckle BC, Geist J (2016). Establishing mussel
9 behavior as a biomarker in ecotoxicology. *Aquatic Toxicology* **170**, 279–288.
- 10 Hasler CT, Hannan KD, Jeffrey JD, Suski CD (2017). Valve movement of three species
11 of North American freshwater mussels exposed to elevated carbon dioxide.
12 *Environmental Science and Pollution Research* **24**, 15567–15575.
- 13 Higgins PJ (1980). Effects of food availability on the valve movements and feeding
14 behavior of juvenile *Crassostrea virginica* (Gmelin). *Journal of Experimental Marine*
15 *Biology and Ecology* **46**, 17–27.
- 16 Jakšić Ž, Batel R, Bihari N, Mičić M, Zahn RK (2005). Adriatic coast as a microcosm
17 for global genotoxic marine contamination - A long-term field study. *Marine Pollution*
18 *Bulletin* **50**, 1314–1327.
- 19 Jenner HA, Noppert F, T S (1989). A new system for the detection of valve movement
20 response of bivalves. *Kema Scientific and Technical Reports* **7**, 91–98.
- 21 Johannesson K, Smolarz K, Grahn M, André C (2011). The future of baltic sea
22 populations: Local extinction or evolutionary rescue? *Ambio* **40**, 179–190.
- 23 Klarić S, Pavičić-Hamer D, Lucu Č (2004). Seasonal variations of arsenic in mussels
24 *Mytilus galloprovincialis*. *Helgoland Marine Research* **58**, 216–220.
- 25 Kramer KJM (2009). Continuous monitoring of waters by biological early warning
26 systems. In: Gonzalez C, Greenwood R, Quevauviller PP, eds. *Rapid Chemical and*
27 *Biological Techniques for Water Monitoring*. John Wiley & Sons Ltd, Chichester, pp.
28 197–219.
- 29 Kramer KJM, Foekema EM (2000). The “Musselmonitor®” as biological early warning
30 system. In: Butterworth FM, Gunatilaka A, Gensebatt ME, eds. *Biomonitoring and*
31 *Biomarkers as Indicators of Environmental Change 2: A Handbook*. Springer, New
32 York, pp. 59–87.

- 1 Kuwatani Y (1963). Effect of photo-illumination on rhythmical movement of pearl
2 oyster, *Pinctada martensii* (Dunker). *Bulletin of the Japanese Society of Scientific*
3 *Fisherie* **29**, 1064–1070.
- 4 Langton RW (1977). Digestive rhythms in the mussel *Mytilus edulis*. *Marine Biology*
5 **41**, 53–58.
- 6 Lummer EM, Auerswald K, Geist J (2016). Fine sediment as environmental stressor
7 affecting freshwater mussel behavior and ecosystem services. *Science of the Total*
8 *Environment* **571**, 1340–1348.
- 9 Mizuta DD, Wikfors GH (2019). Depth selection and in situ validation for offshore
10 mussel aquaculture in Northeast United States federal waters. *Journal of Marine*
11 *Science and Engineering* **7**, 1–32.
- 12 Nagai K, Honjo T, Go J, Yamashita H, Seok JO (2006). Detecting the shellfish killer
13 *Heterocapsa circularisquama* (Dinophyceae) by measuring bivalve valve activity with a
14 Hall element sensor. *Aquaculture* **255**, 395–401.
- 15 Niedda M, Pirastru M, Amponsah W, March L, Zoccatelli D, Marra F et al. (2015). The
16 cyclone Cleopatra of November 18, 2013 in Sardinia, event management , measurement
17 and modelling. *Quaderni di Idronomia Montana* **32**, 47–58.
- 18 Pavičić J, Raspor B, Martinčić D (1993). Quantitative determination of metallothionein-
19 like proteins in mussels. Methodological approach and field evaluation. *Marine Biology*
20 **115**, 435–444.
- 21 Petrović S, Ozretić B, Krajnović-Ozretić, M Bobinac D (2001). Lysosomal membrane
22 stability and metallothioneins in digestive gland of mussels (*Mytilus galloprovincialis*
23 Lam.) as biomarkers in a field study. *Marine Pollution Bulletin* **42**, 1373–1378.
- 24 Polsenaere P, Soletchnik P, Le O, Gohin F, Robert S, Pépin J et al. (2017). Potential
25 environmental drivers of a regional blue mussel mass mortality event (winter of 2014,
26 Breton Sound, France). *Journal of Sea Research* **123**, 39–50.
- 27 Qiu JW, Tremblay R, Bourget E (2002). Ontogenetic changes in hyposaline tolerance in
28 the mussels *Mytilus edulis* and *M. trossulus*: implications for distribution. *Marine*
29 *Ecology Progress Series* **228**, 143–152.
- 30 Rajagopal S, Van Der Velde G, Jenner HA (1997). Shell valve movement response of
31 dark false mussel, *Mytilopsis leucophaeta*, to chlorination. *Water Research* **31**, 3187–
32 3190.

- 1 Rao KP (1954). Tidal rhythmicity of rate of water propulsion in *Mytilus californianus*
2 and its modifiability by transplantation. *Biological Bulletin* **106**, 283–293.
- 3 Redmond KJ, Berry M, Pampanin DM, Andersen OK (2017). Valve gape behaviour of
4 mussels (*Mytilus edulis*) exposed to dispersed crude oil as an environmental monitoring
5 endpoint. *Marine Pollution Bulletin* **1–2**, 330–339.
- 6 Robson A, Wilson R, De Leaniz CG (2007). Mussels flexing their muscles: A new
7 method for quantifying bivalve behaviour. *Marine Biology* **151**, 1195–1204.
- 8 Robson AA, Garcia De Leaniz C, Wilson RP, Halsey LG (2010). Behavioural
9 adaptations of mussels to varying levels of food availability and predation risk. *Journal*
10 *of Molluscan Studies* **76**, 348–353.
- 11 Rovero F, Hughes RN, Chelazzi G (1999). Cardiac and behavioural responses of
12 mussels to risk of predation by dogwhelks. *Animal Behaviour* **58**, 707–714.
- 13 Rovero F, Hughes RN, Whiteley NM, Chelazzi G (2000). Estimating the energetic cost
14 of fighting in shore crabs by noninvasive monitoring of heartbeat rate. *Animal*
15 *Behaviour* **59**, 705–713.
- 16 Seggel A, De Young C (2016). *Climate Change Implications for Fisheries and*
17 *Aquaculture - Summary of the Findings of the Intergovernmental Panel on Climate*
18 *Change Fifth Assessment Report*. Rome.
- 19 Tran D, Fournier E, Durrieu G, Massabuau JC (2004). Copper detection in the Asiatic
20 clam *Corbicula fluminea*: optimum valve closure response. *Aquatic Toxicology* **66**,
21 333–343.
- 22 Turolla E (2016). *Gasteropodi e Bivalvi Marini Dei Mercati Europei Volume 2*
23 *(Arcidae, Glycymerididae e Mytilidae)*. Tipografia Giari, Italy, Codigoro.
- 24 Underwood AJ (1997). *Experiments in Ecology: Their Logical Design and*
25 *Interpretation Using Analysis of Variance*. Cambridge University Press, Cambridge.
- 26 Viarengo A, Canesi L (1991). Mussels as biological indicators of pollution. *Aquaculture*
27 **94**, 225–243.
- 28 Wilson R, Reuter P, Wahl M (2005). Muscling in on mussels: New insights into bivalve
29 behaviour using vertebrate remote-sensing technology. *Marine Biology* **147**, 1165–
30 1172.
- 31 Zippay ML, Helmuth B (2012). Effects of temperature change on mussel. *Integrative*
32 *Zoology* **7**, 312–327.

- 1
2
3
4 1 Zuur AF, Ieno EN, Smith GM (2007). *Analysing Ecological Data*. Springer, New York.
5
6 2 de Zwart D, Kramer KJM, Jenner HA (1995). Practical experiences with the biological
7
8 3 early warning system “mosselmonitor”. *Environmental Toxicology and Water Quality*
9
10 4 **10**, 237–247.
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

Parameter	Concentration (ppm)
Nitrate (NO ₃ -)	21.352
Nitrite (NO ₂ -)	0.007
Silicate (SiO ₂)	0.378
Total Phosphate	0.010

3
4

For Review Only

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
<i>P</i>	< 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.

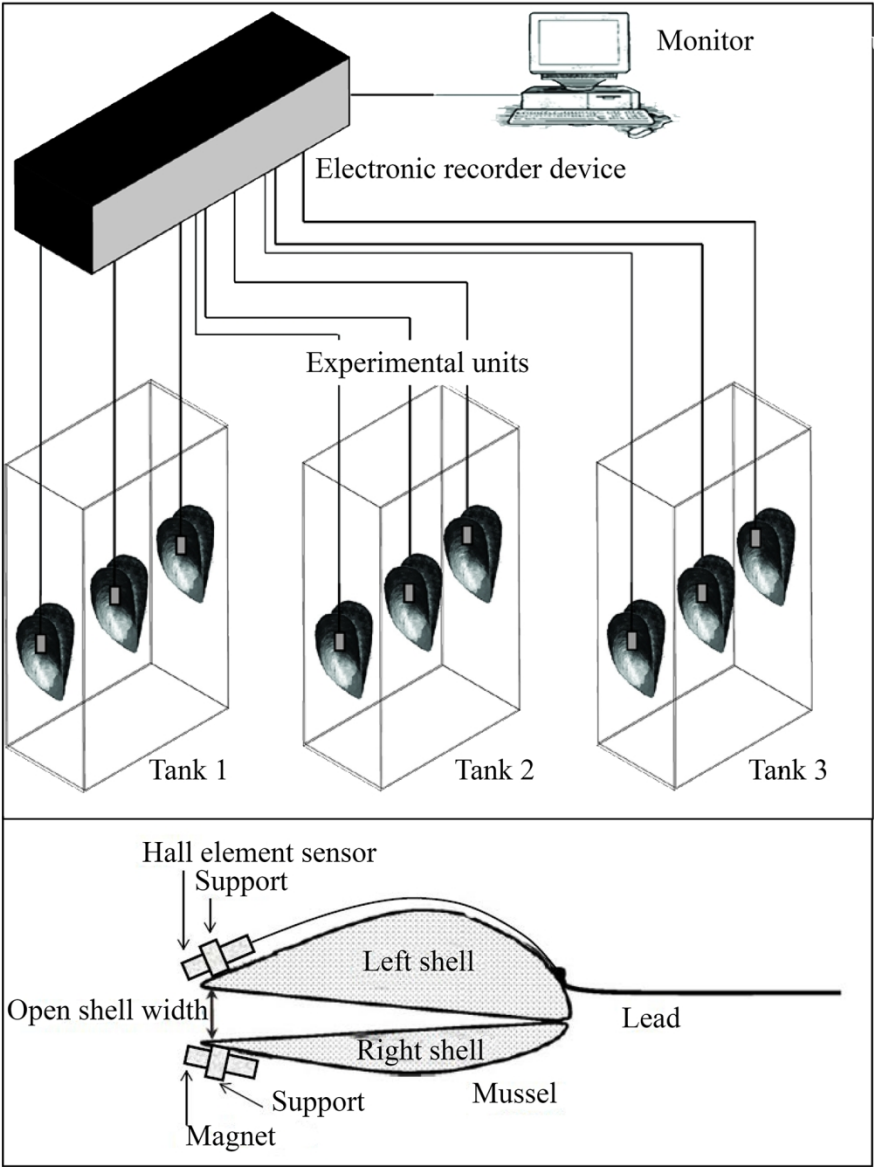


Figure 1 Scheme of the valvometer device utilized for the experiment (above), and detail of the connection of the Hall's sensor-magnet to mussel valves (below).

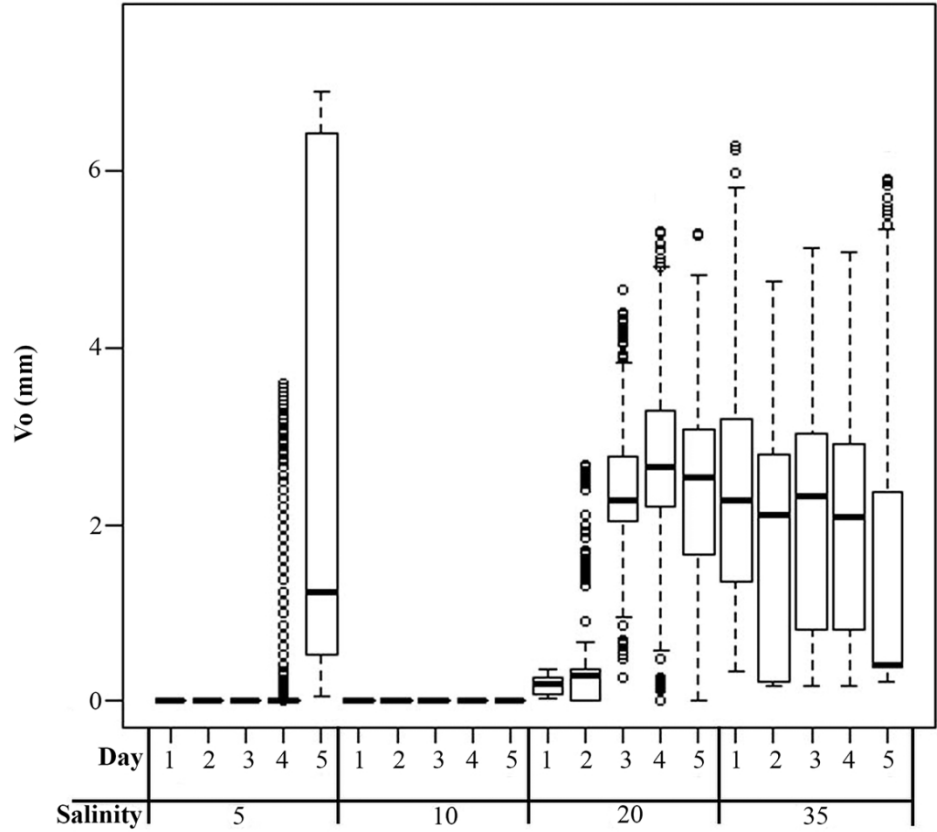


Figure 2 Box plots of the valve gaping (Vo) for three class of salinity (5, 10 and 20 PSU) and the reference salinity (35 PSU) during recordings of day 1-5.

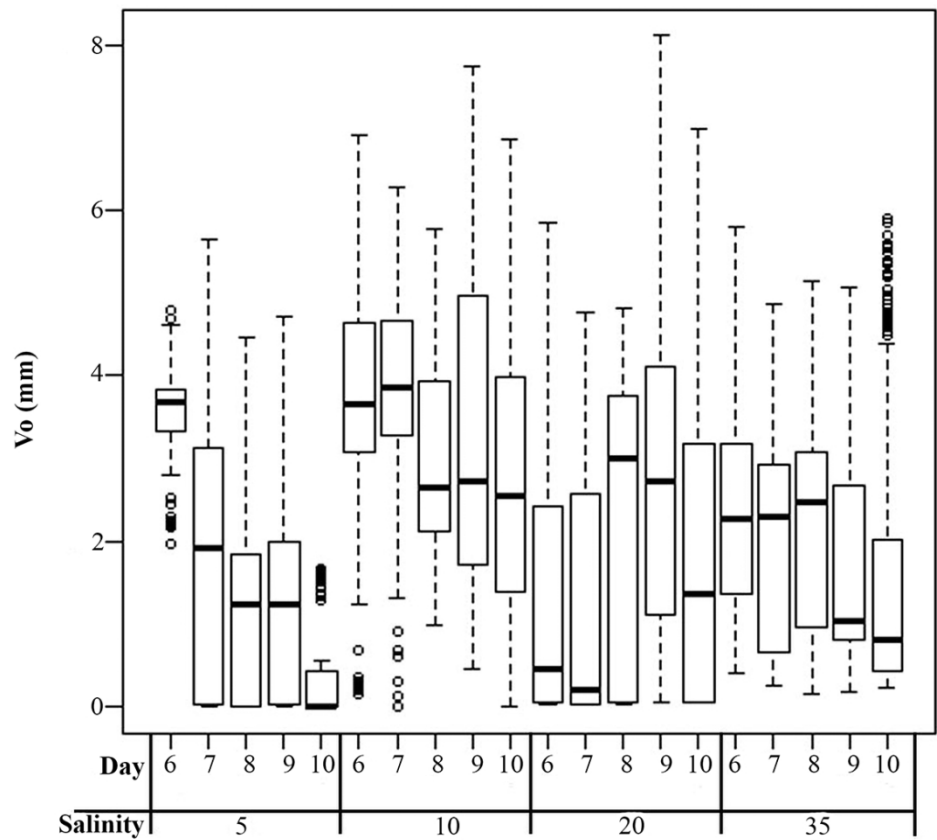


Figure 3 Box plots of the valve gaping (Vo) for three class of salinity (5, 10 and 20 PSU and the reference salinity (35 PSU) during recordings of day 6-10 when the reference state was re-established. Vo at salinity of 5 is referred two survived mussels.

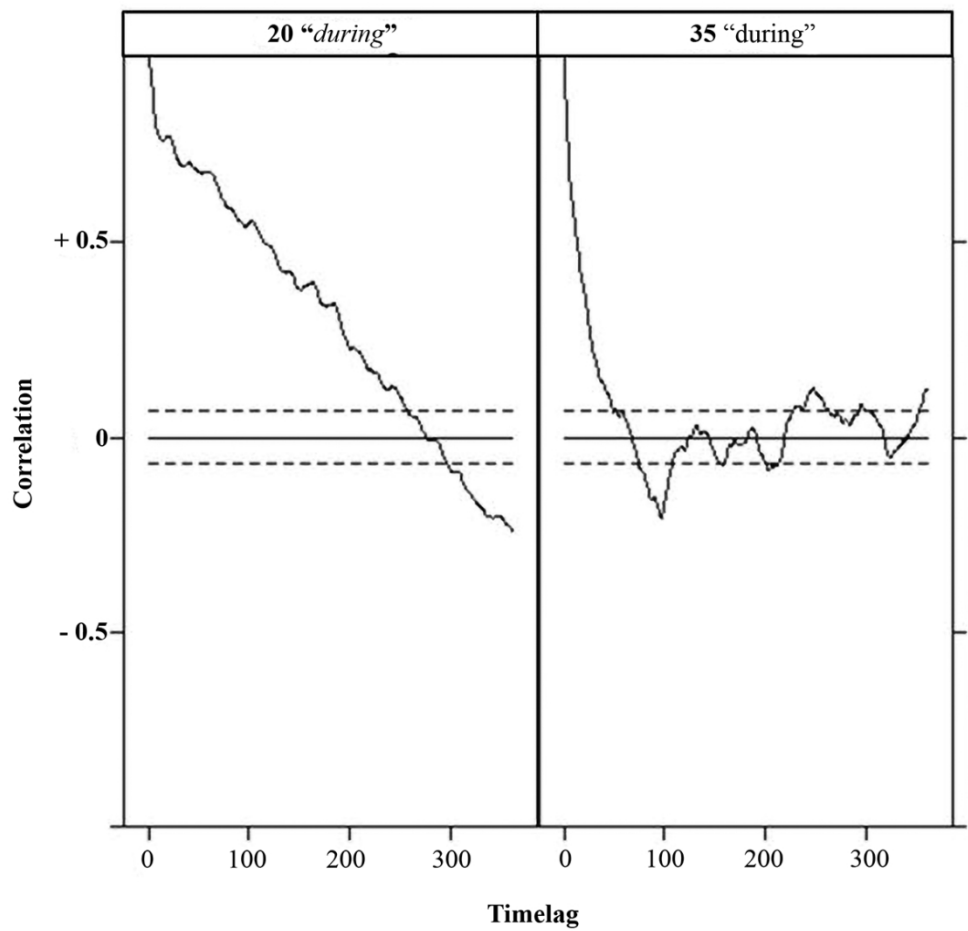


Figure 4 Autocorrelation function (ACF) for valve gaping (Vo) recordings at salinity 20 and 35 PSU considering the During exposure.

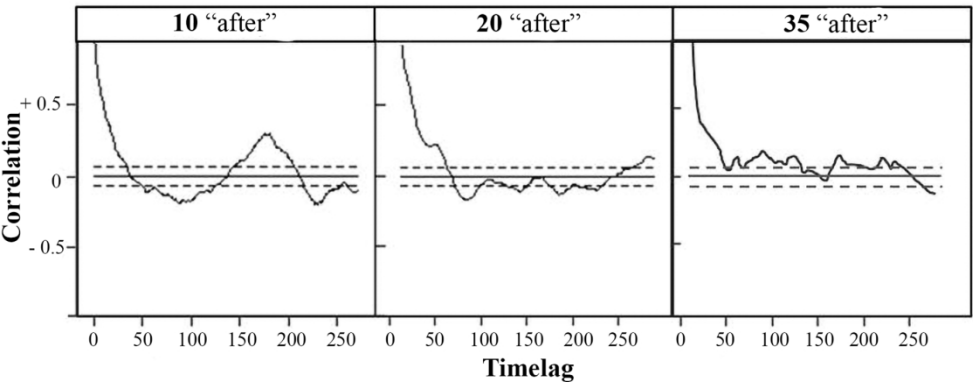


Figure 5 Autocorrelation function (ACF) for valve gaping (Vo) recordings at salinity 10, 20 and 35 PSU considering the After exposure.

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 to 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 epoxy 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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

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.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

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 influences of environmental 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 know, 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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

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