Tolerance to hypoxia in Asian green mussels, *Perna viridis*, collected from a ship hull in the non-native range in eastern Indonesia

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Abstract

Tolerance to fluctuating environmental conditions is regarded as a key trait of successful marine invasive species as it presumably promotes survival in recipient habitats, which are often anthropogenically impacted systems such as harbours. Little is known, however, about how transport of fouling organisms on ship hulls influences the condition of the transported individuals and how this is related to their tolerance to environmental stress. We investigated the influence of transport on a ship hull on the ability of Asian green mussels, *Perna viridis*, to survive low concentrations of dissolved oxygen (0.5 and 1 mg/l DO). This was done by comparing the performance under stress in mussels from a eutrophic habitat in Jakarta Bay to that of mussels that had spent their lifetime on a passenger ferry crossing the Indonesian Archipelago from Jakarta in the west to West Papua in the east. We found that the mussels that came from the eutrophic habitat survived twice as long as mussels from the ferry when exposed to low oxygen concentrations. Mussels collected from the ferry, however, had a generally higher byssus production under experimental conditions, which can be attributed to their life on a moving object where they are exposed to drag. We suggest that Jakarta Bay mussels survived oxygen stress longer because they had higher Body Condition Indices than their conspecifics from the ship hull and thus had more energy available for stress compensation. These results show that transport on ship hulls can weaken the robustness of *P. viridis*, if the journey leads the ship through areas of low food supply for mussels, if the stopovers in eutrophic coastal ecosystems are short and if the sailing times are long (several weeks). This finding might explain the lack of establishments of *P. viridis* in tropical areas of Australia, from where repeated incursions have been reported.

Key words: hull fouling, hypoxia, stress tolerance, nutritional status, Indonesian archipelago

Introduction

The introduction of non-native species into areas from which they were previously absent is problematic as introduced species may compete with native biota and alter ecosystem structure and function (Kimbro et al. 2013; Mack et al. 2000). However, not every introduced species also establishes and spreads, i.e. becomes invasive. Invasion success depends on the condition of the receiving habitat, the timing of the introduction, propagule pressure, i.e. the number of reproductive units introduced, and on the species’ life history traits (Van Kleunen et al. 2010).

Life history traits often found in widespread invasive marine invertebrates are: having a good ability to disperse as adults (e.g. by fouling on hard substrates) or during long phases as pelagic larvae, high fecundity, fast growth and a high tolerance towards changing environmental conditions (Sakai et al. 2001). Some of these traits may, however, also...
differ between individuals and populations of the same species, for instance if local adaptations have caused differences between populations from different origins or if momentary health conditions differ between individuals. Several studies compared the performance of native and non-native species co-occurring in the same habitat and found that the non-native species often had a higher tolerance to fluctuating environmental conditions (i.e. abiotic stress) than the native species (Bielen et al. 2016; Jewett et al. 2005; Lenz et al. 2011; Schneider 2008).

In the present study, we investigated the ability of the successful invader *Perna viridis* (Linnaeus, 1758) to survive extreme environmental conditions (hypoxia) as a function of the habitat where the respective individuals spent their life: in a eutrophic coastal ecosystem in the western, native range of the species in Indonesia, or as hull fouling on a ferry in the eastern, non-native range of the species in Indonesia. Testing the tolerance to environmental stress in marine invertebrates that were transported as hull fouling and that may establish a founder population outside the native range of the species presumably helps to understand why not all incursions of non-native species result in biological invasions (or establishment). Individuals may benefit from a good stress tolerance if introduced to a habitat that is anthropogenically impacted, as it is often the case in harbours with high ship traffic. Piola and Johnston (2006), for example, investigated copper (Cu) tolerance in the invasive bryozoan, *Bugula neritina*, that established on ship hulls coated with Cu-containing antifouling paint and found a higher copper resistance in colonies that originated from a Cu-polluted harbour (Piola and Johnston 2006).

Despite this example, no study has ever compared tolerance to environmental stress in a population of marine invertebrates that was sampled in a benthic habitat in the species’ native range, to tolerance in a conspecific population that was found on a ship hull in the non-native range. However, such an approach should shed light on the question whether individuals undergo changes in their health and nutritional status during transport. These changes might reduce or enhance their potential to establish and spread after arrival. To investigate this, we chose hypoxia as an environmental stressor.

As with other mytilids, *P. viridis* responds to hypoxia by closing its valves and by down-regulating its metabolism (Wang et al. 2011). Under these conditions, the mussels fuel their life processes from their energy reserves, which are stored as glycogen in their body tissues. The more energy in the form of glycogen is available, the longer a mussel individual will be able to survive (Fearman et al. 2009). We therefore suggest hypoxia tolerance as a suitable indicator of the physical status of *P. viridis*, which also allows conclusions on *P. viridis*’ potential to establish at a non-native site.

Furthermore, hypoxia is a common stressor in highly impacted habitats, such as large harbours, which, at the same time, are those habitats with the highest number of potentially arriving propagules. Examples where hypoxic conditions, high frequencies of ship traffic and the existence of dense *P. viridis* populations coincide are Tolo harbour, Hong Kong (Cheung 1993), Kingston harbour, Jamaica (Buddo et al. 2003), Singapore (Holmes et al. 1999) and Jakarta Bay, Indonesia (Wendling et al. 2013). Under these conditions, hypoxia tolerance can be critical for the survival of a newly arriving mussel population. Even in well oxygenated waters, however, mussels that have ample energy reserves should have a better chance to establish than mussels with depleted reserves as this would, for example, extend the maximum time of remaining closed during predator exposure. However, apart from tolerance to hypoxia, there are several other factors that could determine the potential to establish in introduced populations of *P. viridis*. The data presented here add to the results of a study that used the same mussel populations to investigate potential dispersal routes of *P. viridis* in Indonesia and which discusses the potential of establishment as a function of the mussels’ nutritional and reproductive status (Huhn et al. 2015).

**Materials and methods**

**Mussel collection and acclimatization**

Two separate laboratory experiments compared tolerance to hypoxia between mussels from a native population in the eutrophic Jakarta Bay (JB), West Java, Indonesia, and mussels that fouled the hull of the ferry KM Tidar (TI) at Banda Naira, Moluccas, in the non-native range in Indonesia. At the day of collection, sea surface temperatures (SST) and dissolved oxygen (DO) concentrations at the collection sites were 30.3 °C and 3.99 mg/l in Jakarta Bay (31st May 2014) and 29.4 °C and 6.54 mg/l at Banda Naira (15th November 2013). The mussels collected from Muara Kamal in JB had a shell length (SL) of 42.5–50.0 mm with a mean SL = 45.7 mm), were tested in the marine habitat laboratory at Bogor Agricultural University (IPB), which is a 3–4 hour drive from the collection site. During transport, the mussels were kept dry in an insulation box. The experiment with the mussels from the ferry (SL: 30.2–45.7 mm, mean SL = 34.6 mm) was conducted in a laboratory at Banda Naira, where the mussels...
were collected from the sheltered leeward inside of the ferry’s front propeller slot (Huhn et al. 2015). Here, the mussels were transferred to acclimatization tanks directly after collection as the laboratory is located only 100 m away from the harbour.

At both sites, the salinity of the water in which the mussels were kept during acclimatization was the same as at the collection site since the water came from these sites. Water temperatures, however, were slightly lower in the tanks than in the field: 27°C in the laboratory in Bogor and 28 °C in the laboratory at Banda Naira. Before the experiments, mussels were cleaned of epibionts and were acclimatized to laboratory conditions for 5 days (JB mussels) and 14 days (TI mussels) since, due to logistical restrictions, acclimatization times could not be standardized across sites. All other variables (amount and type of food, water exchange rates, aeration and mussel-to-water ratios) were the same at both sites. During acclimatization, mussels were kept in tanks with 80 L of aerated seawater at a density of 1 mussel per liter and were fed with 0.01 ml of the highly concentrated filter feeder food Sera Marin Coralliquid® (SMC) per individual per day. A 50% water exchange was conducted daily.

BCI determination

Immediately after acclimatization, before the beginning of the hypoxia experiment, subsamples with n = 20 mussels per population were removed from the tanks and immediately frozen. Individuals from Banda Naira were later transported to IPB by plane in an insulation box on ice and the mussels from Jakarta Bay were frozen at IPB until further processing. Body condition indices (BCIs), as a measure for the nutritional status, were determined by separating the soft tissue from the shell and drying both at 60 °C for 24 hours. The BCI was then calculated as the dry soft tissue (in g) divided by the dry shell (in g). We tested for a possible relationship between mussel sizes (JB = 45.7 mm, TI = 34.6 mm mean SL) and BCI by plotting the BCIs of Jakarta Bay mussels, which were collected between April 2012 and November 2013 (n = 324), as a function of their shell lengths. As in the range of 30–60 mm BCIs did not change with shell length, we assumed that mussel size will not bias our results (Figure 1, Supplementary material Table S1). BCI data were tested for statistically significant differences between populations with the non-parametric Wilcoxon rank sum test because the data could not be transformed to normality.

To obtain information about a possible change in the mussels’ BCIs during acclimatization to the laboratory, we calculated the ratios of BCIs before and after acclimatization. The BCIs (mean ± sd) of KM Tidar mussels after collection were 0.092 ± 0.014 (n = 58, data published in Huhn et al. 2015). As we do not have BCI data of the mussels that were collected in Jakarta Bay on May 31st 2014, we used the average BCIs of mussels collected from the same site between April 2012 and November 2013 (data published in Huhn et al. 2016a). The BCIs of these mussels were 0.223 ± 0.075 (mean ± sd, n = 364).

Exposure to different concentrations of dissolved oxygen

For the experiment in which mussels were exposed to different concentrations of dissolved oxygen (DO), 45 adult individuals per population (mean SL ± sd: JB = 45.4 ± 1.8 mm and TI = 34.5 ± 2.1 mm) were singled out and placed in PVC containers filled with 600 ml of seawater so that one mussel in one container represented one experimental unit. Water in the units of the control group (DO > 6 mg/l, n = 15 per population) was aerated with compressed air using aeration stones. For the experimental groups with lowered oxygen concentrations, water with 0.5 mg/l DO and with 1.0 mg/l DO (Sal = 33, T = 27 °C (Bogor) and Sal = 35, T = 28 °C (Banda)) was prepared in a header tank by using a nitrogen flow from a gas cylinder. When the target oxygen concentration was reached, which was validated by measuring the oxygen concentration with a WTW Oxical 3205 oxygen meter and CellOx 325 sensor, the experimental containers were filled with the respective de-oxygenated water and covered with o-ring sealed lids so that no air space remained in the container. Replication was again 15 mussel individuals per population and oxygen concentration.

The same technique of decreasing the oxygen concentration was used to prepare seawater for the daily water exchange. Experimental conditions were maintained for 14 days, during which mussels were daily fed with 0.01 ml SMC per individual. Every day, mortality was recorded in all containers by carefully touching gaping mussels with a thin stick. Mussels that did not respond to this stimulation were
considered dead and were removed from the experiment. Survival over 14 days was statistically compared between groups with a Coxph survival analysis in the package “Survival” in R (R Development Core Team 2013; Therneau 2013; see Huhn et al. (2016a) for a detailed description of the procedure.

Byssus thread production

To measure byssus formation during exposure to hypoxia, the number of byssus threads produced by each mussel individual was counted after 48 hours under the experimental conditions (0.5, 1.0 and > 6 mg/l DO). For this, byssus terminal discs were marked with a permanent marker on the outside of the transparent PVC container while counting to avoid double counts. Count data were analysed statistically with a generalized linear model (glm) with Poisson distribution in R (R Development Core Team 2013), which included two factors, “Population” (2 levels: Jakarta Bay and KM Tidar) and “Treatment” (3 levels: 0.5, 1.0 and > 6 mg/l DO).

Results

BCIs at Experimental Start and after collection

Before the start of the experiment, BCIs differed significantly between mussels from Jakarta Bay and those collected from the hull of the ferry KM Tidar. They were 2.5 times higher among the mussels from Jakarta Bay and this difference was statistically significant (mean BCIJB = 0.143, mean BCITI = 0.059, Wilcoxon rank sum test: W = 400, p < 0.001, nJB = 20, nTI = 20, Figure 2, Table S2).

The mean ratios of BCIs before to after acclimatization were 0.642 (TI) and 0.640 (JB). This means that mussels in both groups had lost 36% of their soft tissue weight during acclimatization to laboratory conditions.

Survival during exposure to hypoxia

During 14 days of exposure to two levels of hypoxia (0.5 mg/l and 1.0 mg/l DO), mussels from Jakarta Bay and from KM Tidar showed progressing mortality, whereas individuals in the control groups that were maintained under 100% oxygen saturation survived by 87% (JB) and 100% (TI) (Table S3). There was no significant interaction between the factors “Population” and “Oxygen concentration” and the interaction term was removed from the Coxph model. In the simplified model, the factor “Population” was highly significant (p < 0.0001, exp(coef)TI = 3.02, n = 60), while the factor “Oxygen concentration” was marginally significant (p = 0.08, exp(coef)1mg/l =0.62, n = 60).

Thus, the probability to survive hypoxic conditions (both levels) was 202% higher in JB mussels than in TI mussels, while the chance to survive, averaged across both populations, was slightly higher at 1 mg/l DO (38%) than at 0.5 mg/l DO (Figure 3).

Byssus threads

Even though the mussels from the ferry KM Tidar were on average 10 mm smaller than the mussels from Jakarta Bay, they produced more byssus threads.
This was true under all oxygen concentrations (Table 1, Figure 4). The largest difference in mean byssus production between the populations was found at 1 mg/l DO (3.7 times more byssus in TI than in JB mussels), followed by 0.5 mg/l DO (3.2 times more byssus in TI mussels) and normoxia (1.7 times more byssus in TI mussels) (Table S3).

Discussion

The experiment presented here shows that Asian green mussels, *Perna viridis*, that had spent their lifetime on the hull of a ferry, which has short stopover times (2–3 hours at most harbours and maximum 18 hours at Jakarta Bay once in 14 days, Huhn et al. 2015), had a lower tolerance towards two levels of hypoxia than mussels that came from a eutrophic coastal habitat. In contrast to survival, byssus production during the first 48 hours of stress exposure, regardless of oxygen concentration, was higher among mussels that were collected from the ship hull. This result suggests that the environmental conditions experienced by an individual mussel during its lifetime may determine its byssus formation: Mussels that were taken from the ferry hull had presumably acclimated to the prevailing drag and shear stress by forming more byssus threads in order to attach firmly when the ship is moving and maintained this acclimation during the course of our experiment. Furthermore, as all mussels were collected from inside the front propeller slot, they were additionally exposed to strong currents produced by the propeller whenever the ship was manoeuvring sideways (during docking and departing). As these mussels presumably stemmed from various recruitment events, which most likely occurred in different harbours (Huhn et al. 2015), it is unlikely that the higher byssus production resulted from genetic adaptation that could have occurred at a single location of origin, but rather was a consequence of acclimation to life on a moving object. That byssus production is adjusted in response to environmental cues is known, for example, in response to predatory pressure and changes in salinity and temperature (Young 1985; Cheung et al. 2009).

A striking difference between the mussels from JB and TI was their BCI, which, at the start of the experiment, was 2.5 times higher in mussels from JB—the group that also showed the higher tolerance to hypoxia. In a eutrophic habitat, such as Jakarta Bay, there is ample food supply for filter feeders such as mussels (Huhn et al. 2016a), whereas during an open ocean journey on a ship hull, especially in tropical regions, food availability can be scarce, as *P. viridis* mainly feeds on phytoplankton. A proxy for phytoplankton abundance that is applicable at large spatial scales is the surface Chlorophyll *a* (Chl *a*) concentration. In Jakarta Bay, Chl *a* concentrations range from 8 to 93 µg/l (Damar 2003), whereas along the route of the ferry KM Tidar, in the open Java Sea, Flores Sea and Banda Sea, Chl *a* concentrations range from 0.1 to 2.4 µg/l, depending on the season (Kinkade et al. 1997). In mussels, including *Mytilus edulis*, growth rates are most often a positive function of Chl *a* concentrations in the respective habitat (Philippart et al. 2014). This means that for *P. viridis* growth should be by magnitudes higher if the mussels come from a eutrophic environment such as Jakarta Bay than if they live on a ferry that is spending most of the time at sea travelling through oligotrophic areas.

This study shows that the mussels from the ferry had lower energy reserves than their conspecifics from the eutrophic habitat. In mytilids, energy for metabolic processes is stored in the form of glycogen, which is deposited in the mantle tissue, whereas dietary lipids, if not metabolized instantly, are mainly allocated directly to gamete formation (Fearman et al. 2009). Consequently, an insufficient diet, under which all available energy is needed for metabolic processes, would slow down gamete formation and would limit glycogen storage (Fearman et al. 2009). During extended periods of environmental stress, such as hypoxia, stored glycogen can be metabolized by anaerobic respiration, while the valves of the mussel stay closed to isolate the soft body from the environment. Consequently, insufficient glycogen storage should shorten survival time during exposure to adverse conditions. In accordance with this, we found that the mussels that came from KM Tidar had low BCIs, which is a valid proxy for energy reserves (Norkko et al. 2005), did not have any well-developed gonads (Huhn et al. 2015) and showed low survival during hypoxia. Moreover, these findings indicate that the risk that mussel individuals, which fall off the hull in a port outside the distributional range of the species, lead to the establishment and spread of *P. viridis* is low. This should be especially true if the mussels are lacking mature gonads due to an insufficient food supply. The risk of establishment, however, should increase if ships carrying *P. viridis* on their hulls would spend days to weeks in a eutrophic harbour or coastal area where the animals can refill their energy resources. Long stopovers are possible during ship clearings by customs on international shipping routes or during technical inspections.

The experiment presented here highlights the relevance of energy availability for stress compensation and survival in *P. viridis*. However, other mechanisms, which are not related to food supply, such as pre-acclimation to low oxygen concentrations...
in mussels from JB, where hypoxic periods occur regularly (Arifin 2006; Huhn et al. 2016a), may additionally have contributed to the longer survival time among JB mussels. In a previous study, we showed that acclimation to low oxygen concentrations can increase performance under hypoxia in P. viridis (Huhn et al. 2016b), but also found evidence for the significance of high BCIs for mussel survival under low oxygen concentrations. Unfortunately, we could not disentangle potential acclimation effects from the role of energy availability on the base of this experiment, but the large differences in BCIs that we observed (BCIs were 2.5 times higher in JB than in KM Tidar mussels) suggest a significant influence of energy availability on survival during hypoxia. Furthermore, the survival times we observed for the ferry mussels investigated in this study were the lowest of all found in similar experiments that were conducted with P. viridis from 2012 until 2014 (Huhn et al. 2016a; Huhn et al. 2016b). The averaged survival time of adult JB mussels under an oxygen concentration of 0.5 mg/l was 6.8 days in previous experiments (n = 4 experiments), while it was 7.5 days in specimens from less impacted habitats at the coast of West Java (n = 3) (Huhn et al. 2016a, Huhn et al. 2016b). However, the mussels that were collected from the hull of a ferry had a mean survival time of only 2.7 days. This difference corresponds well to the observed difference in BCIs between the mussels from the ferry and from populations along the coast of west Java (Huhn et al. 2015). It is unlikely, that the divergent survival times under hypoxia between JB and ferry mussels that were found in the present study were due to a difference in body size. Since not all size classes were available, the mussels from Jakarta Bay, which were used in this study, were larger than the mussels from the ferry. However, if size would have influenced the outcome of this experiment, it rather should have reduced the effect size as per capita oxygen consumption increases with size. Therefore, large mussels should be more susceptible to hypoxia than smaller conspecifics. In accordance with this, we showed that juvenile P. viridis from Jakarta Bay survived longer under hypoxia (13 mm shell length, 12.6 days survival) than adults from the same site (34 mm, 10 days; 43 mm, 9.5 days; 65 mm, 3.1 days) (Huhn et al. 2016a; Huhn et al. 2016b). Therefore, if size would have been the driver of survival, the smaller mussels from the ferry should have survived longer.

If the environmental conditions that hull fouling individuals of P. viridis experience during an open ocean voyage weaken their robustness, as suggested by the results of this study, this mechanism may be one explanation for the fact that P. viridis never established in Australia, even though frequent incursions of the species have been reported (McDonald 2012, Dr J. McDonald, Western Australian Fisheries, pers. comm). Therefore, the nutritional status of mussels is one factor that should be considered when assessing the establishment potential of ship-transported P. viridis. However, for a better understanding of the influence that transport on ship hulls can have on the performance of individuals of P. viridis after arrival in a new habitat, further studies are needed. They should also consider other potential stressors than hypoxia that occur during a ships’ journey and which may influence the mussels’ nutritional status and robustness (e.g. temperature or salinity stress). To identify these it would be necessary to measure fluctuations in abiotic and biotic variables, such as temperature, oxygen concentration, salinity and phytoplankton abundance along common shipping routes (Wang et al. 2011; Huhn et al. 2016a). In addition to this, small submersible cameras, which can be attached to the ship hull, can be used to monitor activity patterns in mussels to establish, for example, whether the animals are gaping while the ship is moving. Together with data on phytoplankton abundance, recorded gaping times would give valuable information on the potential utilization of food during an ocean voyage. Additionally, it will be necessary to sample more mussels from ship hulls to verify whether the low BCIs and poorly developed gonads found in individuals from the ferry at Banda Naira are representative for ship-transported mussels or are an exception. Studies that directly investigate the performance of hull fouling organisms from ship hulls are so far rare (Murray et al. 2012; Schimanski et al. 2016). More studies on physical conditions following transport as hull fouling would provide valuable information about the potential for establishment and spread in P. viridis and other fouling invertebrates after arrival in their non-native range.

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Tolerance to hypoxia in Asian green mussels


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Supplementary material

The following supplementary material is available for this article:

**Table S1.** Measurements of shell length and content of dry soft tissue for *Perna viridis* from Jakarta Bay and from a ferry hull.

**Table S2.** Body condition indices (BCIs) of *Perna viridis* before the beginning of exposure to hypoxia stress.

**Table S3.** Survival time and byssus thread production in *Perna viridis* during exposure to hypoxia stress.

This material is available as part of online article from: http://www.reabic.net/journals/mbi/2017/Supplements/MBI_2017_Huhn_etal_Supplement.xls