Zoological Studies **56:** 27 (2017)
doi:10.6620/ZS.2017.56-27

Zoological Studies

Open Access

Influence of Water Temperature on the MXR Activity and P-glycoprotein Expression in the Freshwater Snail, *Physa acuta* (Draparnaud, 1805)

Cristina N. Horak and Yanina A. Assef*

CONICET, Centro de Investigaciones Esquel de Montaña y Estepa Patagónicas (CIEMEP), Laboratorio de Investigaciones en Ecología y Sistemática Animal (LIESA), Universidad Nacional de la Patagonia San Juan Bosco, 9200 Esquel, Chubut, Argentina

(Received 4 January 2017; Accepted 8 September 2017; Published 2 October 2017; Communicated by Benny K.K. Chan)

Cristina N. Horak and Yanina A. Assef (2017) P-glycoprotein (P-gp) mediated multixenobiotic resistance (MXR) is a mechanism analogous to multidrug resistance, which has been extensively characterized in mammalian tumours. The expression and function of the MXR mechanism has been demonstrated in numerous aquatic organisms and has been proposed as a biomarker for pollution assessment. A close relationship between thermal stress and MXR response has been reported in some aquatic organisms. Seasonal studies in freshwater organisms are scarce and conducted mainly in zebra mussel (*Dreissena polymorpha*), whose presence has not been reported in South America. The general purpose of the present study was to evaluate seasonal variation of a biomarker, the MXR mechanism, in the worldwide distributed freshwater snail *P. acuta*. We analyzed the *in situ* influence of temperature on the biomarker response over an 18-month field study. MXR defence system was evaluated by a combination of functional assays (RB accumulation) and molecular approaches to analyse P-gp expression. The results demonstrated a linear correlation between MXR response, at activity and expression level, and water temperature at sample site, in *P. acuta* snails. The characterization of the MXR system in worldwide distributed species, including the study of their seasonal fluctuations, could contribute to the increasing interest to incorporate this biomarker to provide an integrated assessment of mussel health status. This work supports the possible use of *P. acuta* snails with this purpose and also highlights that the occurrence of variations in MXR response related to water temperature has to be taken into account in the interpretation of *in situ* monitoring studies.

Key words: MXR, P-glycoprotein, Biomarker, Temperature, Freshwater snail.

BACKGROUND

Biological systems can be monitored for a wide range of goals, including assessing the health of ecosystems, the effects of certain natural or anthropogenic factors of stress and for evaluating sustainability. According to van Gestel and van Brummelen (1996), a biomarker is defined as any biological response to an environmental chemical at the below-individual level, measured inside an organism or in its products, indicating a change compared to the normal state that cannot be detected from the intact organism. However, many more definitions of the concept of biomarker can be found in the literature (Hyne and Maher 2003, Bartell 2006). In ecotoxicological studies, biomarkers can integrate the interactive effects of complex mixtures of chemicals experienced by organisms in ecosystems impacted by different compounds (Fent 2004).

The multixenobiotic resistance (MXR) mechanism found in aquatic organisms represents a transport system of cellular detoxification and have been proposed to play a major role as a first line of cellular defence. MXR is similar to the well-known phenomenon termed multidrug resistance (MDR) involved in mammalian tumour cells resistant to chemotherapeutic drugs (Kurelec 1992; Bard 2000). The pharmacological basis for this resistance appears to be associated with the

*Correspondence: E-mail: yassef@conicet.gov.ar

expression of efflux pumps that are members of the ATP-binding cassette (ABC) transporter family. The P-glycoprotein (P-gp) is the most studied protein of this family and recognizes a wide variety of structurally unrelated compounds, leading to a reduction in intracellular drug accumulation and hence impairing its efficacy (Borges-Walmsley et al. 2003; Loo and Clarke 2005).

Numerous field and laboratory studies strongly indicate that the P-gp mediated MXR activity may critically influence the susceptibility of several aquatic organisms to different xenobiotic agents. In view of the fact that different elements of MXR transport system (verapamil sensitivity of accumulation rate and immunochemical reactivity of P-gp) appear to be proportional to the level of pollution, it was proposed by several authors as a biomarker of exposure (Kurelec et al. 1996; Minier and Moore 1996; Epel 1998; Eufemia and Epel 1998; Smital and Kurelec 1998; Smital et al. 2003). Moreover, the use of MXR mechanism as a biomarker allows monitoring the biological effects on aquatic biota that may be due to a complex mixture of compounds (Minier et al. 2006a).

Since the first observation of the environmental relevance of these transporters, the P-gp related multixenobiotic transporter was mainly described in mussels, sponges, oysters and fish (Kurelec 1992; Kurelec at al. 1996; Smital et al. 2000; Bard 2000, Luckenbach et al. 2014). However, not all of these species express the MXR system as sensitive indicator of contaminant exposure (Damaré et al. 2009; Hamdoun et al. 2002; Assef et al. 2014a).

Several studies conducted in the last years in the aquatic environment field have indicated the need to consider interactions among other biotic and abiotic factors. The seasonal variation of these abiotic factors such as temperature, salinity, turbidity, among others, can influence the interpretation of biomarker responses (Bodin et al. 2004; Pain et al. 2007). In this regard, temperature has been proposed as one of the main regulatory factors of MXR by several authors, as there was a positive trend between MXR activity or P-gp expression and temperature. Various works have reported a close relationship between thermal stress and MXR response in marine organisms such as the common mussel *Mytilus galloprovincialis* and the oyster *Crassostrea virginica* (Keppler and Ringwood 2001b; Minier et al. 2000; Kamel et al. 2014). However, seasonal studies in freshwater organisms are scarce and conducted mainly in the zebra mussel, *Dreissena polymorpha,* whose presence has not been reported in South America (Smital et al. 2003; Pain et al. 2007). The MXR response in zebra mussel was highly correlated with the water temperature in field studies but no significant changes in the MXR expression were observed when bivalves were slowly acclimated to the temperature in aquaria condition (Pain et al. 2007; Tutundjian and Minier 2007).

Recently, the first evidence of the P-gp-like transport system in a freshwater pulmonate snail (*Physa acuta*) present in streams of Patagonia was presented. In that study we demonstrated the deinduction of the MXR activity and P-gp expression when snails were transferred from the polluted site to clean water, suggesting its role as a defence mechanism (Assef et al. 2014b). *P. acuta* is considered as one of the most ubiquitous aquatic macroinvertebrates in the world (Dillon et al. 2002). These snails are particularly suitable for ecotoxicological studies because they are worldwide distributed, abundant in aquatic ecosystems, easy to collect and to handle for laboratory assays. Moreover, their benthic locomotory and feeding habits make them vulnerable to elevated concentration of xenobiotic agents in sediments (Musee et al. 2010).

The general purpose of the present study was to assess seasonal variation of the MXR mechanism in the freshwater snail *P. acuta*. We analyzed the *in situ* influence of temperature on the biomarker response (MXR activity and P-gp expression) over an 18-month field study. These results contribute to the elucidation of the significance of MXR mechanism in *P. acuta* snails and their possible use as a biomarker of aquatic contamination.

MATERIALS AND METHODS

Study area and sampling

The watercourse selected for the study is the Esquel stream that flows through the homonymous city (32758 inhabitants, Instituto Nacional de Estadística y Censos, 2010), in Chubut province, Argentina. This stream is placed in the Futaleufú-Yelcho basin and it is disturbed in its middle section by discharges from the city waste water treatment plant (WWTP). The samplings were all carried out in the same place (42°58'32"S, 71°23'47"W) located at 5.4 km downstream of WWTP. The sampling periods that include autumn,

winter, spring and summer season were between April 2012 and October 2014.

Water temperature was recorded *in situ* on each sampling occasion. Depending on the season at the time of collection the water temperature ranged between 1.0-15.7°C. In all cases, the environmental conditions at the sampling day were representative of at least four days before, where no rainfall or significant changes in ambient temperature were recorded. Conductivity ($\mu S.cm^{-1}$), pH, and dissolved oxygen (mg $O_2.l^{-1}$) were obtained with a multi-parameter probe (Hach SensION 156). Water samples (2 l) were collected below the water surface and kept at 4°C for nutrient analyses. At the laboratory, nitrate plus nitrite-nitrogen, ammonia, soluble reactive phosphate (± 0.1 $\mu g.l^{-1}$) and total suspended solids (± 0.1 $mg.l^{-1}$) were analyzed following the American Public Health Association methods (APHA 1994).

Experimental organisms

Physa acuta, a common pulmonate snail mostly found in streams, ponds, and lakes throughout the world, was used as a sentinel organism. Specimens of the freshwater snail *P. acuta* (shell lengh 9.4 ± 1.4 mm) were collected manually from Esquel stream. Snails were carried alive to the laboratory in water of origin. After collection, all the specimens were maintained in flowing clean dechlorinated water at 8-12°C in the laboratory's glass aquaria. The multixenobiotic defence system was then assessed using the bioaccumulation method within the first 2-6 hours of collection (see Measurement of MXR activity below). Based on *in vitro* studies of the biosynthesis, processing and half-life of P-gp (Yoshimura et al.1989; Zhang and Ling 2000), no change in protein expression is expected within this time. Thus, the MXR activity and P-gp expression levels are representative of the collection day conditions.

For laboratory depuration, a group of 20 specimens was isolated and kept in the aquaria with dechlorinated clean water at 8-12°C for 7 days to confirm the deinduction of protein levels by molecular biology studies. The medium was changed every two days. Snails were given no food during the adaptation period and then were fed *ad libitum* with lettuce leaves.

Measurement of MXR activity

The *in vivo* bioaccumulation assay is based on the measurement of the accumulation level of P-gp substrate, the fluorescent dye Rhodamine B (RB), in aquatic organisms after the exposure to the dye without (control) and with addition of verapamil, a model MXR inhibitor. This assay was performed as previously published assays to obtain the clearest results in *Physa acuta* snails; this included the determination of optimal incubation times, as well as the working concentration of RB and verapamil (Assef et al. 2014b). Animals were placed into a Petri dish (5 snails/plate) containing 50 ml of dechlorinated tap water supplemented with 5 μM RB (Sigma, St. Louis, MO, USA) in absence or presence of 30 μM verapamil (Sigma). Snails were exposed to the corresponding medium for 4 h. Incubation of snails in a medium containing RB enabled the probe to cross the cellular membranes by passive diffusion and to accumulate within the organism. After the exposure period, snails were washed three times in 150 ml of dechlorinated tap water using a tea strainer. The entire body from each specimen was gently isolated from the shell using a dental explorer probe. Bodies from five specimens were weighed, transferred to 1.5 ml centrifuge tube containing 0.5 ml of distilled water and homogenised for 30 s (Pro200 Homogenizer, Pro Scientific Inc., USA). Homogenates were centrifuged at 3000 x *g* for 5 min and the supernatants carefully transferred to clean tubes. The RB-fluorescence of supernatants was measured immediately (each supernatant in duplicate) using a fluorometer (QuantiFluor-TM, Promega, USA). Data represents the accumulated dye in the entire body of five snails. The fluorescence values were provided in concentration units and data were expressed in picomol of accumulated RB per gram of organism ($pmol.g^{-1}$).

We used the *R*-value as the primary criterion for the quantification of the level of MXR activity (Smital et al. 2000). The *R*-value can be determined by the ratio between the amount of RB accumulated into the organisms in control condition, and the amount of dye accumulated in presence of verapamil. Thus, the theoretically maximal *R*-value is 1 while the minimal value tends to 0 (that means theoretically maximum of MXR activity).

The assay was carried out not before 2 h of collection, the time required for the release of possible environmental pollutants (MXR substrates) previously bound on active sites of P-gp (Kurelec et al. 2000). During the procedure all material was light protected to avoid the possible

loss of the RB fluorescence intensity caused by direct exposure to light.

Protein extraction

The entire body from specimens of *P. acuta* were gently isolated, washed twice in dechlorinated clean water, transferred to 1.5 ml centrifuge tubes in 0.5 ml of lysis buffer (250 mM NaCl, 50 mM HEPES, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail; Sigma) to prepare total protein extracts. The mixtures were subjected to homogenisation for 15 s on ice (Pro200 Homogenizer, Pro Scientific Inc., USA), then centrifuged at 6000 x *g* for 10 min at 4°C and the supernatants carefully transferred to clean tubes. Total protein in each sample was quantified according to Bradford method and stored at -20°C until use. Each homogenate represents total protein extracts from five specimens (pooled samples).

Western blot analysis

Total protein extracts were dissolved in loading buffer (50 mM Tris, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 100 mM DTT, and 0.2 mg/ml bromophenol blue). The heating step was omitted to minimise membrane protein aggregation. Then, 50 μg of total protein was separated by 8% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membrane (Hybond ECL, Amersham, GE Healthcare, Buckinghamshire, UK). Five μl of molecular weight marker (Promega) was also migrated. The optimal incubation conditions were previously described (Assef et al. 2014b). Briefly, the membrane was blocked with 10% dry milk in Tris–saline buffer containing 0.05% Tween 20 (T-TBS) and then incubated with P-gp specific C219 monoclonal antibody (Calbiochem, USA) diluted 1:1000 for 3 h at room temperature (RT) or α-tubulin (Santa Cruz Biotechnology, Inc., CA, USA) as housekeeping gene product, diluted 1:5000 for 1 h at RT. The membranes were washed five times with T-TBS and then incubated with horse anti-mouse Immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) diluted 1:5000 for 1 h at RT. After washing five times with TBS, positive reactivity was detected using the ECL Western Blotting Analysis System (Amersham, GE Healthcare, Buckinghamshire, UK) and the chemiluminescence reaction was visualised on AGFA Medical X-Ray films (Agfa-Gevaert S.A., Argentina). The relative level of each protein was obtained using the ImageJ 1.37v densitometric software (National Institutes of Health, USA).

Dot blot

Protein extracts prepared as described above were applied to a nitrocellulose membrane (2 μl per dot) for dot blot analysis. Immunoassays were conducted following the protocol of Galgani et al. (1996) using C219 as the primary antibody. P-gp was detected using the same primary and secondary antibodies as for the Western blot procedure. Dots were revealed by the chromogenic substrate 3, 3′-diaminobenzidine tetrahydrochloride (DAB, Sigma). The dot blots were digitalized and quantified using image analysis software. Results are expressed as the amount of P-gp (in intensity units) per total protein content determined according to Bradford method.

Statistical analysis

The resulting data were analyzed separately using a variety of statistical tools according to the type of experiment. Data are given as mean ± standard deviation (SD). Mean values were calculated from the results obtained for at least five groups of 5 specimens per condition, and *n* indicates the number of groups used in each experiment. Data statistical analysis was performed using one-way analysis of variance (ANOVA) followed by pos hoc test when significant (Duncan's test). A paired Student's t-test was used for comparison the level of P-gp bands between control and depurated extracts. Differences were considered significant when $p < 0.05$. Spearman correlations were computed to investigate linear relationship between biomarker values (MXR activity or P-gp expression) and physical or chemical stressors, where r_s indicates the Spearman coefficient. In order to compare the strength of the linear relationships between the MXR activity and water temperature, we measured the effect size that reflects the practical meaningfulness of the relationship among variables. The standardized estimates of $r_s > 0.5$ for a strong effect, $r_s \approx 0.3$-0.5 for a moderate effect, and $r_s \approx 0.1$-0.3 for a weak effect were considered (Cohen 1988; Maher et al. 2013).

RESULTS

Seasonal variation of MXR activity

The activity of the MXR system was evaluated in the *Physa acuta* snails as function of the seasons by way of the bioaccumulation of RB method. We incubated the specimens in the medium with 5 μM of RB in the absence or presence of the MXR inhibitor verapamil. Figure 1A shows that the MXR transporter was effective at exporting the fluorescent probe in all cases. Accumulations of RB significantly increased in the presence of high concentrations of verapamil (30 μM) in comparison with the corresponding control group of animals ($p < 0.05$; $n = 7$). The lowest control accumulation of RB occurred in autumn (853.9 ± 89.3 pmol RB.g^{-1}) being significantly different than the other seasons ($p < 0.05$). The accumulation in presence of the inhibitor was similar for all four seasons. Calculated *R*-values indicate no variations in the MXR activity between seasons (Fig. 1B).

Effect of temperature on MXR activity

In order to study whether MXR activity is related to temperature, measurements of water temperature were made at the sampling sites and the bioaccumulation assays in *P. acuta* snails were performed monthly. Maximum water temperatures were reached during the months of December to March, with values close to 17°C, while in July the minimum records were obtained (Fig. 2). Although

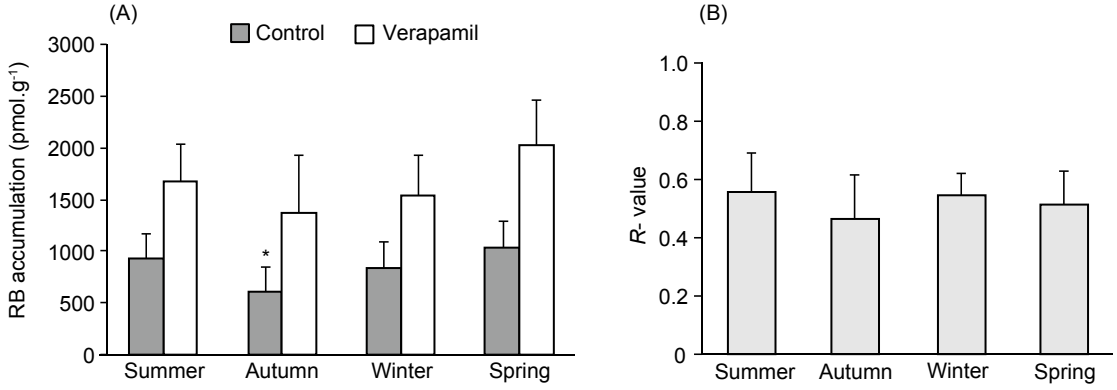

Fig. 1. Measurement of MXR activity in *Physa acuta* snails according to seasons. (A) The accumulation of RB has been assessed in control snails and after inhibition with 30 μM verapamil. Data are expressed in picomoles of accumulated RB per gram of entire snail body weight. The symbol (*) indicates a significant difference compared to the other bars in control condition ($p < 0.05$, $n = 7$). (B) Bars represent the *R*-value calculated from the data present in the panel A (control /verapamil). In all cases, bars represent standard deviation of the mean.

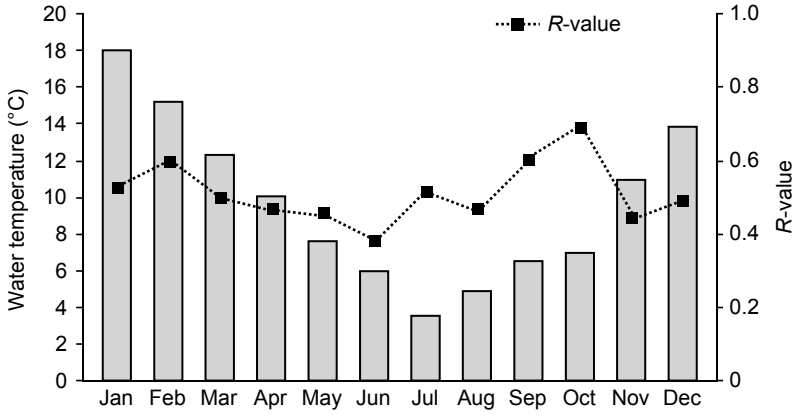

Fig. 2. Seasonal variation of water temperature at sampling site. Bars represent the average value of water temperature recorded each month over an 18-month field study. The accumulation of RB has been also assessed in the presence and absence of 30 μM verapamil for each month. The calculated *R*-values (control /verapamil) were plotted on the accessory y-axis. No significant variations were observed between R-values ($p > 0.05$).

MXR activity, expressed as *R*-values, was more heterogeneous throughout the months of September and October, no significant variations were observed, appearing not following the seasonal pattern of average water temperature.

To further assess the effect of temperature on MXR activity we analyzed the correlation between each RB bioaccumulation data with the water temperature corresponding to the day of collection, regardless of the period of the year. Figure 3 showed a significant negative linear correlation between the MXR response and water temperature (r_s = -0.48, p = 0.013; n = 26).

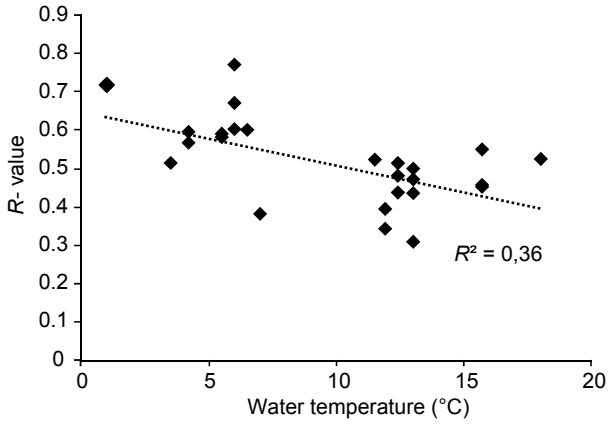

Fig. 3. Representation of *R*-values as a parameter to determine the MXR activity in *P. acuta* snails versus water temperature. *R*-value negatively correlates with water temperature (Spearman r_s = 0.48, p = 0.013; n = 26).

Immunochemical detection of P-gp

The expression of P-glycoprotein on *P. acuta* tissue homogenates was assessed by Western blot analysis using the monoclonal antibody C219 directed toward a highly conserved epitope in the C-terminal region. The C219 antibody recognised a diffuse band at ~170 kDa and a major reactive band that appears at 220-240 kDa, as reported previously for *P. acuta* snails (Assef et al. 2014b). Figure 4A, shows the mean relative levels of each specific immunoreactive bands obtained by densitometric analysis. α-tubulin was used as internal control. Following the depuration period of 7 d, the expression of both immunoreactive bands was reduced. Similar results for both bands were obtained when the level of P-gp was quantified in function of total protein content using Ponceau S (data not shown).

The C219 antibody was also used for dot blot experiments on *P. acuta* tissue homogenates. The antibody staining increased with increasing concentration of total proteins in the samples. The analysis of the intensity of each dot using densitometric measurements indicates that the assay is reliable in the range of 0.25 to 7 μg.μl^{-1} of protein concentration with a linear curve on a

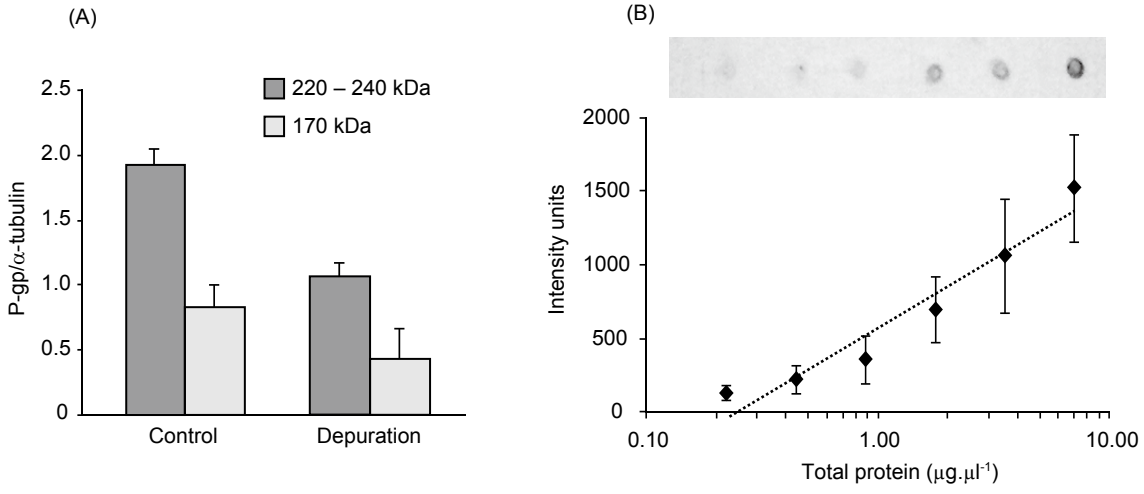

Fig. 4. Inmunodetection of P-gp in *P. acuta* snails using the anti-P-gp C219 antibody. (A) Expression of ~170 kDa and 220-240 kDa bands of P-gp was analysed on collection day and following a depuration period of 7 days by Western blot. The relative level of P-gp using α-tubulin as the internal control is represented in the bar graph. Data are given in intensity units and represent a mean ± SD from three separate experiments. (B) Calibration curve for dot blot analysis using C219 antibody. Upper panel: labeling of monoclonal antibody to increasing amounts of total protein from *P. acuta* homogenates (0.22, 0.44, 0.88, 1.75, 3.50 and 7.00 μg.μl^{-1} of protein). Bottom panel: Standard curve of intensity of the label for each dot. Data represent a mean ± SD from six separate experiments. The X-axis (μg.μl^{-1} of proteins) is on a log scale.

semilog scale (Fig. 4B). The detection limit of this technique in our experimental condition is 0.25 ± 0.08 $\mu g.\mu l^{-1}$.

Effect of temperature on P-gp expression

The correlation between the P-gp levels with the water temperature was analyzed. Figure 5 showed a significant positive linear correlation between the intensity unit per dot and water temperature (r_s = 0.47, p = 0.002, n = 8).

As water temperature is probably not the only parameter involved in MXR and P-gp variations, main environmental features of the sampled reach such conductivity, dissolved oxygen, pH, nutrients and total suspended solids, have been measured during this study. These physical and chemical parameters were also tested against the MXR activity and P-gp expression. Table 1 shows the correlation coefficients and their associated probabilities between the biological responses and these parameters. We have not found a significant correlation between MXR activity or P-gp levels and pH, dissolved oxygen, conductivity, total suspended solids and several nutrients.

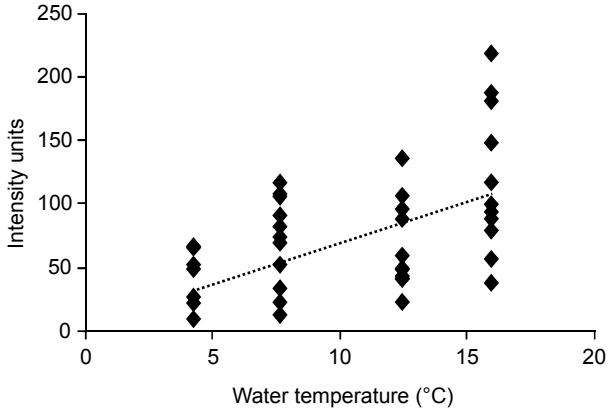

Fig. 5. Representation of P-gp expression (intensity units per total protein content) in *P. acuta* snails versus water temperature. P-gp levels in total snail homogenates positively correlates with water temperature (Spearman r_s = 0.47, p = 0.002, n = 8).

DISCUSSION

Several works have reported a close relationship between thermal stress and MXR response in marine molluscs (Bodin 2004; Kamel et al. 2014; Minier et al. 2000), but the information about freshwater species is limited (Pain et al. 2007; Tutundjian and Minier 2007). The screening of different aquatic organisms has been proposed to find those species in which MXR activity can result less influenced to seasonal variations. In this regard, benthic animals might be less susceptible to seasonal stresses than mussels that are typically harvested from littoral areas (Minier et al. 1999). The present study analysed the seasonal variation of a biomarker, the MXR mechanism, in the worldwide distributed freshwater snail *P. acuta* inhabiting an urban stream of Patagonian Andes. Whereas most previous studies were based on expression levels of P-gp, in this study the MXR defence system was evaluated by a combination of functional assays and molecular approaches.

We determined the accumulation of RB in animals collected in autumn, winter, spring and summer. In all cases, the accumulation of RB dye observed immediately after collection resulted significantly increased after the addition of a transport inhibitor, verapamil. The lowest control value of accumulation of RB in *P. acuta* occurred in autumn. Minier et al. (2000) found that the MXR protein expression levels in gills of the marine mussel *Mytilus galloprovincialis* appeared to follow the pattern of water temperature but delayed

Table 1. Correlation coefficients (r_s) between MXR biomarker responses (activity and P-gp expression) and chemical or physical variables

	MXR activity	P-gp expression
pH	-0.208 (0.457; 15)	0,002 (0.991; 8)
Dissolved oxygen ($mg.l^{-1}$)	-0.315 (0.253; 15)	-0,039 (0.813; 8)
Conductivity ($\mu S.cm^{-1}$)	-0.121 (0.668; 15)	-0,002 (0.991; 8)
Total suspended solids ($mg.l^{-1}$)	-0.182 (0.644; 9)	-0,039 (0.813; 8)
Nitrate plus nitrite-nitrogen ($\mu g.l^{-1}$)	-0.417 (0.177; 12)	0,231 (0.151; 8)
Soluble reactive phosphate ($\mu g.l^{-1}$)	-0.118 (0.716; 12)	-0,002 (0.991; 8)
Ammonia ($\mu g.l^{-1}$)	0.374 (0.228; 12)	-0,272 (0.090; 8)

The associated probabilities and the number of determinations are in brackets.

with respect to the warmest season. However, when *R*-values were calculated, which include data recorded in the presence and absence of verapamil, no variations in the MXR activity were observed between seasons. Similarly, the pattern of monthly water temperature is not accompanied with variations in the activity of MXR.

The ranges of water temperatures are very wide during certain months in the mountain streams of Patagonia (Miserendino 2001, Miserendino et al. 2011). In view of this, we decided to further assess the effect of temperature on the MXR activity by analyzing a correlation between each MXR activity data and the corresponding water temperature record. We found a significant linear correlation between the MXR activity and water temperature. Based on the value of the absolute coefficient (r_s = -0.48), a moderate effect size was found between these variables suggesting a biological relevance of this observation (Maher et al. 2013). As the *R*-value was used as a parameter to evaluate the biomarker response, the correlation has a negative slope. These results were in agreement with previous field studies that found that Rhodamine B transport activity in zebra mussel was highly correlated with water temperature (Pain et al. 2007). However, no significant change in MXR activity nor in P-gp expression was recorded when mussels were maintained at laboratory-controlled temperatures ranging from 4 to 20°C (Tutundjian and Minier 2007).

We also studied the MXR response at P-gp expression level since most seasonal previous studies were based exclusively on its measurements (Minier et al. 2000; Keppler and Ringwood 2001a, b; Bodin et al. 2004; Kamel et al. 2014). P-gp expression has been previously observed in *P. acuta* homogenates, characterized by the presence of only two immunoreactive bands at ~170 kDa and > 220 kDa and there was no cross-reactivity elsewhere on the blot (Assef et al. 2014b). Similar multiple banding has also been observed in the gill tissue of some mussels and oysters species (reviewed in Bard 2000). An unresolved question is whether the larger molecular weight bands are indeed transport molecules (Galgani et al. 1996). It has been reported that C219 antibodies cross-react with a ~200 kDa band corresponding to the heavy chain of muscle myosin (Thiebaut et al. 1989). However, C219 did not bind to myosin in protein samples from several aquatic organisms (Keppler and Ringwood 2001a; Damaré et al. 2009). Recently, a full-length cDNA sequence of the P-gp was successfully cloned from *Mytilus californianus* and *Chlamys farreri* encoded polypeptides with the predicted molecular mass close to 150 kDa (Luckenbach and Epel 2008; Miao et al. 2014). Glycosylation most likely explains the discrepancy between the predicted size from the amino acid sequence and the size indicated for this protein in Western blot analyses (Luckenbach and Epel 2008). Interestingly, Whalen et al. (2010) found a typical band of expected size, 170 kDa and two larger diffuse bands, >200 kDa, possibly representing differentially glycosylated isoforms of P-gp or the presence of multiple proteins in the marine mollusc *Tritonia hamnerorum.* A depuration of experimental organisms in clean water is often performed to assess the background or baseline level of MXR transporters (Smital and Kurelec 1997; Smital et al. 2000; Pain and Parant 2007). After a depuration period, the expression of both bands in *P. acuta* samples was reduced suggesting its involvement in the MXR phenotype and also showing the specificity of the antibody. Therefore, dot blot technique was used to quantify P-gp expression from the seasonal study. Western blot is the most common method for evaluating and quantifying protein expression; however, it is very labor intensive and time consuming when dealing with large sample sizes (Putra et al. 2014). Dot blot have been previously used instead of Western blot analyses for P-gp in aquatic organisms (Galgani et al. 1996; Keppler and Ringwood 2001b; Minier et al. 2006b). This technique allows a good reproducibility and permits that a larger number of samples could be analyzed in less time.

Our results confirmed the observations presented above based on functional studies. The expression levels of P-gp in *P. acuta* homogenates is positive correlated with the water temperature according with results in marine species (Minier et al. 2000; Keppler and Ringwood 2001a; Kamel et al. 2014). Indeed, *Mytilus californianus* mussels exposed to heat shock of 20 or 25°C exhibited increased P-gp levels and activity compared to mussels held at ambient (12°C) temperature seawater (Eufemia and Epel 2000). They also showed that several of the stressors causing P-gp induction also caused a concomitant induction of heat shock protein 70 (hsp70), which are involved in cellular protein homeostasis and repair, and are present in all organism studied to date including aquatic invertebrates (De Jong et al. 2008, Stekhoven et al. 2015). This suggests that P-gp induction may be part of a general cellular stress response in mussels.

As only about 40% of the variance can be explained on a linear relationship hypothesis, water temperature is probably not the only parameter involved in MXR and P-gp variations. For example, snails were found to reduce P-gp expression when transferred to clean water for 7 days, suggesting an induction of MXR mechanism in organism inhabiting this sampling site independent of the temperature (Fig. 4A). Moreover, *P. acuta* collected from a relatively unpolluted site showed a significant increase in the accumulation of RB under control conditions (Assef et al. 2014b). In this sense, we have not found a significant correlation between MXR activity or P-gp levels and pH, dissolved oxygen, conductivity, total suspended solids and several nutrients that could explain the enhancement of biomarker in the warmest periods. These results could indicate that the contamination level at the selected site was relatively constant throughout the study period. Accordingly, Minier et al. (2000) showed that no significant correlation existed between MXR and dissolved oxygen or salinity in marine mussel tissues. Anyway, these results do not rule out that other parameters, which might be connected to temperature (*i.e.* food availability, seasonal algal blooms, among others), could be involved in the MXR induction.

Biological effects techniques have been used with the aim to further integrate biological effects measurements with chemical analysis and apply these methods to provide an assessment of aquatic ecosystem health status (Damiens et al. 2007; Moore et al. 2004). In Patagonia, several freshwater species, such as fish, amphibians and amphipods, have shown biochemical and morphological responses to pesticide exposure in both laboratory and field assays (Ferrari et al. 2007; Venturino et al. 2003). A recent study highlights the complexity of biological responses after pesticides exposure and supports the necessity of the integrated use of biomarker for assessing exposure episodes in agricultural areas (Rosenbaum et al. 2012). However, no integrated biomonitoring study conducted in Patagonia has included the MXR as a biomarker of exposure. A straightforward relationship between biomarker response to pollutants at individual or higher biological levels is difficult to establish. In this sense, the knowledge about new species able to be used for these purposes is a key point, especially in poorly studied regions regarding integral biomonitoring. The characterization of the MXR system in worldwide distributed species, including the study of their seasonal fluctuations, could contribute to the increasing interest in incorporating this biomarker to provide an integrated assessment of mussel health status, as proposed previously by other authors (Brooks et al. 2009; Kamel et al. 2014; Minier et al. 2006a). In fact, the OSPAR Strategy applied the integrated biomarker response index (IBR) in order to interpret a global response from the various measured biomarkers, including the MXR as general response marker (ICES 2010).

CONCLUSIONS

The results presented show an increment in the expression and activity of the MXR mechanism in *P. acuta* snails with the water temperature. This information is essential in order to considerer this globally distributed species as an *in vivo* monitor in future studies. Although we found a moderate correlation between the biological response and temperature, any interpretation of these biological parameters should be made with caution in environmental studies.

Acknowledgment: We would like to thank Lic. Mariel Boquet for fieldtrip assistance. Thanks also to the anonymous reviewers for constructive suggestions which greatly improved the manuscript. This work has been supported in part by the National Research Council of Argentina (CONICET; Project Res No 1735/11 and the PIP 11220080101907). This is Scientific Contribution No. 117 from LIESA.

Authors' contributions: CH performed the main field work. CH and YA carried out the laboratory assays and interpreted and analyzed the data. YA contributed to the concept and design and obtained a funding source. Both authors participated in revising the manuscript and approved the final version.

Competing interests: CH and YA declare that they have no conflict of interest.

Availability of data and materials: The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

REFERENCES

APHA. 1994. Standard Methods for the Examination of Water and Wastewater. American Public Health Association. Hanover, Maryland, USA.

Assef YA, Miserendino ML, Horak CN. 2004a. The multixenobiotic resistance mechanism in species of macroinvertebrates associated to an urban stream in the Patagonia mountain. Water Air Soil Poll **225:**1-13.

Assef Y, Horak C, Boquet M. 2014b. Characterization of the multixenobiotic resistance (MXR) mechanism in the freshwater snail *Physa acuta* from Patagonia (Argentina). New Zeal J Mar Fresh **48:**86-96.

Bard SM. 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquat Toxicol **48:**357-389.

Bartell SM. 2006. Biomarkers, bioindicators, and ecological risk assessment - a brief review and evaluation. Environ Bioindic **1:**60-73.

Bodin N, Burgeot T, Stanisière JY, Bocquené G, Menard D, Minier C, Boutet I, Amat A, Cherel Y, Budzinski H. 2004. Seasonal variations of a battery of biomarkers and physiological indices for the mussel *Mytilus galloprovincialis* transplanted into the northwest Mediterranean Sea. Comp Biochem Phys C **138:**411-427.

Borges-Walmsley MI, McKeegan KS, Walmsley AR. 2003. Structure and function of efflux pumps that confer resistance to drugs. Biochem J **376:**313-338.

Brooks S, Lyons B, Goodsir F, Bignell J, Thain J. 2009. Biomarker responses in mussels, an integrated approach to biological effects measurements. J Toxicol Environ Health A **72:**196-208.

Cohen J. 1988. Statistical PowerAnalysis for the Behavioral Sciences, 2nd ed., Hillsdale, NJ: Lawrence Erlbaum.

Damaré CL, Kaddoumi AK, Baer KN. 2009. Investigation of the multixenobiotic resistance mechanism in the freshwater fishes western mosquitofish, *Gambusia affinis*, and Bluegill Sunfish, *Lepomis macrochirus*. B Environ Contam Tox **83:**640-643.

Damiens G, Gnassia-Barelli M, Loquès F, Roméo M, Salbert V. 2007. Integrated biomarker response index as a useful tool for environmental assessment evaluated using transplanted mussels. Chemosphere **66:**574-583.

De Jong L, Moreau X, Thiéry A. 2008. Expression of heat shock proteins as biomarker tool in aquatic invertebrates: Actual knowledge and ongoing developments for the early detection of environmental changes and ecological risks. In Heat Shock Proteins: New Research pp. 375-392.

Dillon RT, Wethington AR, Rhett JM, Smith TP. 2002. Populations of the European freshwater pulmonate *Physa acuta* are not reproductively isolated from American *Physa heterostropha* or *Physa integra*. Invertebr Biol **121:**226-234.

Epel D. 1998. Use of multidrug transporters as first lines of defense against toxins in aquatic organisms. Comp Biochem Phys A **120:**23-28.

Eufemia N, Epel D. 1998. The multixenobiotic defense mechanism in mussels is induced by substrates and non-substrates: implications for a general stress response. Mar Environ Res **46:**401-405.

Eufemia N, Epel D. 2000. Induction of the multixenobiotic defense mechanism (MXR), P-glycoprotein, in the mussel *Mytilus californianus* as a general cellular response to environmental stresses. Aquat Toxicol **49:**89-100.

Fent K. 2004. Ecotoxicological effects at contaminated sites. Toxicology **205:**223-240.

Ferrari A, Venturino A, Pechen de D'Angelo AM. 2007. Muscular and brain cholinesterase sensitivities to azinphos methyl and carbaryl in the juvenile rainbow trout *Oncorhynchus mykiss*. Comp Biochem Physiol C **146:**308-313.

Galgani F, Cornwall R, Toomey B, Epel D. 1996. Interaction of environmental xenobiotics with a multixenobiotic defense mechanism in the bay mussel *Mytilus galloprovincialis* from the coast of California. Environ Toxicol Chem **15:**325-331.

Hamdoun AM, Griffin FJ, Cherr GN. 2002. Tolerance to biodegraded crude oil in marine invertebrate embryos and larvae is associated with expression of a multixenobiotic resistance transporter. Aquat Toxicol **61:**127-40.

Hyne RV, Maher WA. 2003. Invertebrate biomarkers: Links to toxicosis that predict population decline. Ecotoxicol Environ Saf **54:**366-374.

ICES. 2010. Report of the ICES Advisory Committee. ICES Advice. Books 1-11.

Kamel N, Burgeot T, Banni M, Chalghaf M, Devin S, Minier C, Boussetta H. 2014. Effects of increasing temperatures on biomarker responses and accumulation of hazardous substances in rope mussels (*Mytilus galloprovincialis*) from Bizerte lagoon. Environ Sci Pollut Res Int **21:**6108-23.

Keppler CJ, Ringwood AH. 2001a. Expression of P-glycoprotein in southeastern oysters, *Crassostrea virginica*. Mar Environ Res **52:**81-96.

Keppler C, Ringwood AH. 2001b. Expression of P-glycoprotein in the gills of oysters, *Crassostrea virginica*: seasonal and pollutant related effects. Aquat Toxicol **54:**195-204.

Kurelec B. 1992. The multixenobiotic resistance mechanism in aquatic organisms. Crit Rev Toxicol **22:**23-43.

Kurelec B, Krca S, Lucic D. 1996. Expression of multixenobiotic resistance mechanism in a marine mussel *Mytilus galloprovincialis* as a biomarker of exposure to polluted environments. Comp Biochem Phys C **113:**283-289.

Kurelec B, Smital T, Pivcevic B, Eufemia N, Epel D. 2000. Multixenobiotic resistance, P-glycoprotein, and chemosensitizers. Ecotoxicology **9:**307-327.

Loo TW, Clarke DM. 2005. Recent progress in understanding the mechanism of P-glycoprotein-mediated drug efflux. J Membrane Biol **206:**173-185.

Luckenbach T, Epel D. 2008. ABCB- and ABCC-type transporters confer multixenobiotic resistance and form an environment-tissue barrier in bivalve gills. Am J Physiol Regul Integr Comp Physiol **294:**R1919-R1929.

Luckenbach T, Fischer S, Sturm A. 2014. Current advances on ABC drug transporters in fish. Comp Biochem Physiol C **165:**28-52.

Maher JM, Markey JC, Ebert-May D. 2013. The other half of the story: effect size analysis in quantitative research. CBE Life Sci Educ **12:**345-51.

Miao J, Cai Y, Pan L, Li Z. 2014. Molecular cloning and characterization of a MXR-related P-glycoprotein cDNA in scallop *Chlamys farreri*: transcriptional response to benzo(a)pyrene, tetrabromobisphenol A and endosulfan. Ecotox Environ Safe **110:**136-142.

Minier C, Moore MN. 1996. Rhodamine B accumulation and MXR protein expression in mussel blood cells: effects of exposure to vincristine. Mar Ecol Prog Ser **142:**165-173.

Minier C, Eufemia N, Epel D. 1999. The multi-xenobiotic resistance phenotype as a tool to biomonitor the environment. Biomarkers **4:**442-54.

Minier C, Borghi V, Moore M, Porte C. 2000. Seasonal variation of MXR and stress proteins in the common mussel, *Mytilus galloprovincialis*. Aquat Toxicol **50:**167-176.

Minier C, Abarnou A, Jaouen-Madoulet A, Le Guellec AM, Tutundjian R, Bocquené G, Leboulenger F. 2006a. A pollution-monitoring pilot study involving contaminant and biomarker measurements in the Seine Estuary, France, using zebra mussels (*Dreissena polymorpha*). Environ Toxicol Chem **25:**112-119.

Minier C, Moore M, Galgani F, Claisse D. 2006b. Multixenobiotic resistance protein expression in *Mytilus edulis*, *M. galloprovincialis* and *Crassostrea gigas* from the French coasts. Mar Ecol Prog Ser **322:**143-154.

Miserendino ML. 2001. Macroinvertebrate assemblages in Andean Patagonian rivers and streams: environmental relationships. Hydrobiologia **444:**147-158.

Miserendino ML, Casaux R, Archangelsky M, Di Prinzio CY, Brand C, Kutschker AM. 2011. Assessing land-use effects on water quality, in-stream habitat, riparian ecosystems and biodiversity in Patagonian northwest streams. Sci Total Environ **409:**612-624.

Moore MN, Depledge MH, Readman JW, Leonard DRP. 2004. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutat Res **552:**247-68.

Musee N, Oberholster PJ, Sikhwivhilu L, Bothad AM. 2010. The effects of engineered nanoparticles on survival, reproduction, and behaviour of freshwater snail, *Physa acuta* (Draparnaud, 1805). Chemosphere **81:**1196-1203.

Pain S, Devin S, Parant M. 2007. Biomarker versus environmental factors: Seasonal variations and modelling multixenobiotic defence (MXD) transport activity in transplanted zebra mussels. SciTotal Environ **373:**103-112.

Pain S, Parant M. 2007. Identification of multixenobiotic defence mechanism (MXR) background activities in the freshwater bivalve *Dreissena polymorpha* as reference values for its use as biomarker in contaminated ecosystems. Chemosphere **67:**1258-1263.

Putra SE, Tsuprykov O, Von Websky K, Ritter T, Reichetzeder C, Hocher B. 2014. Dealing with large sample sizes: comparison of a new one spot dot blot method to western blot. Clin Lab **60:**1871-1877.

Rosenbaum EA, Duboscq L, Soleño J, Montagna CM, Ferrari A, Venturino A. 2012. Response of biomarkers in amphibian larvae to *in situ* exposures in a fruit-producing region in North Patagonia, Argentina. Environ Toxicol Chem **31:**2311-2317.

Smital T, Kurelec B. 1997. Inhibitors of the multixenobiotic resistance mechanism in natural waters: *in vivo* demonstration of their effects. Environ Toxicol Chem **16:**2164-2170.

Smital T, Kurelec B. 1998. The activity of multixenobiotic resistance mechanism determined by rhodamine B-efflux method as a biomarker of exposure. Mar Environ Res **46:**443-447.

Smital T, Sauerborn R, Pivcevic B, Krca S, Kurelec B. 2000. Interspecies differences in P-glycoprotein mediated activity of multixenobiotic resistance mechanism in several marine and freshwater invertebrates. Comp Biochem Phys C **126:**175-186.

Smital T, Sauerborn R, Hackenberger B. 2003. Inducibility of the P-glycoprotein transport activity in the marine mussel *Mytilus galloprovincialis* and the freshwater mussel *Dreissena polymorpha*. Aquat Toxicol **65:**443-465.

Stekhoven FMAHS, van der Velde G, Lee TH, Bottrill AR. 2015. Proteomic study of the brackish water mussel *Mytilopsis leucophaeata*. Zool Stud **54:**22. doi:10.1186/s40555-014-0081-8.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. 1989. Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein. J Histochem Cytochem **37:**159-64.

Tutundjian R, Minier C. 2007. Effect of temperature on the expression of P-glycoprotein in the zebra mussel, *Dreissena polymorpha*. J Therm Biol **32:**171-177.

van Gestel CA, van Brummelen TC. 1996. Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. Ecotoxicology **5:**217-25.

Venturino A, Rosenbaum E, Caballero de Castro A, Anguiano OL, Gauna L, Fonovich de Schroeder T, Pechen de D'Angelo AM. 2003. Biomarkers of effect in toads and frogs. Biomarkers **8:**167-186.

Whalen K, Sotka E, Goldstone J, Hahn M. 2010. The role of multixenobiotic transporters in predatory marine molluscs as counter-defense mechanisms against dietary allelochemicals. Comp Biochem Physiol C Toxicol Pharmacol **152:**288-300.

Yoshimura A, Kuwazuru Y, Sumizawa T, Ikeda S, Ichikawa M, Usagawa T, Akiyama S. 1989. Biosynthesis, processing and half-life of P-glycoprotein in a human multidrug-resistant KB cell. Biochim Biophys Acta Gen Subj **992:**307-314.

Zhang W, Ling V. 2000. Cell-cycle-dependent turnover of P-glycoprotein in multidrug-resistant cells. J Cell Physiol **184:**17-26.