Portuguese native *Artemia parthenogenetica* and *Artemia franciscana*
survival under different abiotic conditions

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ABSTRACT

There are currently only two places in Portugal where native *Artemia parthenogenetica* can still be found. All other known populations have been eradicated by the invasive species *A. franciscana*, which has caused great losses of *Artemia* biodiversity in the Mediterranean region. The diploid strains found at the Portuguese salines are therefore of high conservation value. This study aims to assess the survival of these native *A. parthenogenetica* strains and of *A. franciscana* under a variety of environmental conditions. The effects of water temperature and salinity, of photoperiod and food supply (shortage) were studied in an experimental setup.

The Portuguese parthenogenetic *Artemia* populations showed great variability in their physiological response to different abiotic conditions, suggesting possible local adaptations in response to different selective pressures experienced. For most of the

conditions studied *A. franciscana* outcompeted the *Artemia* strain from Aveiro, whereas the strain from Rio Maior was more resistant than the *A. franciscana* under conditions that were similar to its local habitat. Strain-specific resistance to chemical conditions, related to pollution, are appointed as a potential cause why *A. franciscana* did not successfully invade Aveiro saline. The saline of Rio Maior has possibly not yet been invaded due to the fitness of its local *Artemia* strain in combination with its inland location.

Keywords: *Artemia*, salinity, temperature, food supply, photoperiod, salines, Portugal.

1. Introduction

Artemia is a widely distributed (Triantaphyllidis et al., 1998) and much studied crustacean. Nine reproductively isolated *Artemia* species have been described, which can be divided into sexual or parthenogenetic species with different ploidy levels (Browne and Bowen, 1991). The genus' large distribution success throughout a variety of environments is evidence of its great adaptability and environmental tolerance (Barata, 1994).

One of the problems currently affecting *Artemia* is its loss of biodiversity in the Mediterranean basin (Amat et al., 2007). There has been a decline of several native *Artemia* populations, due to the introduction of *Artemia franciscana* (Amat et al., 2005), an invasive strain with greater sexual competence, either in terms of its reproductive period or in terms of the number and quality of its offspring (Amat et al., 2007). Amat et al. (2007) compared the sexual fitness of different strains from different environments and parts of the world, under controlled and similar conditions

and observed enormous advantages of *Artemia franciscana* over other strains. Several studies (Browne, 1988; Browne et al., 1991; Wear and Haslett, 1986) show that *Artemia franciscana* resists much better to variations in temperature than other *Artemia* species. Browne and Wanigasekera (2000) also suggest that *A. franciscana* is both euryhaline and eurythermal since it reproduced at a range of salinity/temperature combinations in their experiments. According to Ruebhart et al. (2008), these and several other characteristics strongly contribute to the invasive success of *A. franciscana*. Their work provides extensive information on the worldwide expansion of this species, which is currently present in all continents where the *Artemia* genus is described. The invasiveness and success of *A. franciscana* are very important as they affect endemic lineages and native *Artemia* species.

In Portugal, there are only two places where *Artemia parthenogenetica diploid* exists: the Rio Maior salines (39°21'47"N8°56'33"W) and the Aveiro salines complex (40°38'37"N8°39'57"W) (Pinto et al., 2012). These sites are ± 150 km apart. The Rio Maior saline is an inland saline, supplied by brine from a long and deep streak of rock salt (Calado and Brandão, 2009). In contrast, the Aveiro salines are located in an estuary lagoon, near the Atlantic coast and are hence supplied by sea water (Vieira et al., 1989). The ionic proportion of sea water are often considerably different from the inland salines and salt lakes, that are the places where the *A. parthenogenetica diploid* can usually be found in the Mediterranean basin (Amat, 1980; Barata et al., 1996a, 1996b; Browne and MacDonald, 1982; Zhang and Lefcort, 1991).

In terms of adaption to abiotic conditions, parthenogenetic diploid strains appear to be better adapted to environments with moderate salinities and

temperatures, and do not perform particularly well under extreme temperatures (Amat et al., 1995). In their experiment of the combined effect of temperature and salinity on several *Artemia* strains, Browne and Wanigasekera (2000) found that *A. parthenogenetica* has low phenotypic plasticity when compared to sexual species of *Artemia*. There are however reports of *A. parthenogenetica* populations surviving temperatures of 34°C to 36°C and salinities of 155ppt to 204ppt (Basil et al., 1987). These wide ranging results show that one *Artemia* population may be only representative of its own characteristics (Browne, 1992).

Many studies on *Artemia* biology (e.g. Amat, 1985), ecology (Torreterra, 2004) and population dynamics (e.g. Arashkevich et al., 2009) have been published. Some assess the influence of several factors such as light (e.g. Nambu et al., 2004; Villamizar et al., 2011), salinity (e.g. Vanhaecke et al., 1984; Dana and Lenz, 1986; Browne and Wanigasekera, 2000; El-bermawi et al., 2004; Litvinenko et al., 2007; Agh et al., 2008; Mejía et al., 2009), temperature (e.g. Browne et al., 1988; Barata et al., 1996a, 1996b; Saygi and Demirkaip, 2002) and the type or amount of available food (e.g. Sick, 1976; Evjemo and Olsen, 1999; Lora-Vilchis, 2004) on the survival and reproductive success of different strains and populations of *Artemia*.

Although the *A. parthenogenetica* populations from Aveiro and Rio Maior belong to the same strain and the *Artemia* genus is known to have poor morphological differentiation between populations, strains may show a wide variation in survival and reproductive characteristics, probably as a result of different selective pressures suffered in their original habitats (Browne and Bowen, 1991). The variation in physiological tolerance and life history traits between different *A. parthenogenetica*

populations is sometimes as big as the differences between different *Artemia* species (Browne, 1992). Possible genetic differences between *A. parthenogenetica diploid* populations, caused by different selective pressures experienced may increase the probability of variation in their physiological characteristics (Persoone and Sorgeloos, 1980; Vanhaecke et al., 1984).

The two studied *A. parthenogenetica diploid* populations are of high conservation value, because they are the only known native *Artemia* populations present in Portuguese salines that do not (yet) suffer from invasion, unlike most populations in hypersaline environments of the Mediterranean Basin (Amat et al., 2007). To understand why these particular populations have resisted invasion, a broad picture of the differences between the two native populations and the invasive species *A. franciscana* is needed. The present study was set up to determine the impact of environmental factors on their survival, assessing temperature and salinity effects, which have been widely studied in other parthenogenetic *Artemia* populations (e.g. Barata et al., 1996a, 1996b; Browne et al., 2000), as well as the effects of the amount of provided food (e.g. Sick, 1976; Evjemo and Olsen, 1999; Lora-Vilchis, 2004) and the photoperiod (e.g. Nambu et al., 2004; Villamizar et al., 2011), which are less well studied. We expected to find distinct impacts of these factors on the different *Artemia*, suggesting clues to why the two specific Portuguese native parthenogenetic strains have not been outcompeted and eradicated by *A. franciscana*.

2. Materials and methods

2.1. *Artemia* populations

Artemia parthenogenetica diploid was obtained from samples of adult individuals in the salines of Rio Maior (RM) and Senitra, Aveiro (AV) (Figure 1). Collected individuals were maintained in the laboratory to acclimatize to the following conditions: 24°C temperature, 70ppt salinity, $\pm 300\,000$ cells ml⁻¹ of *Tetraselmis suecica* as food supply and a 12:12h L:D (light:darkness) photoperiod. After obtaining enough cysts from these populations to start the experiment, the cysts were hatched and 300 nauplii were immediately separated for each experimental treatment (starting experimental time). Analogously, *A. franciscana* (AF) were hatched from a commercial brand of *Artemia* cysts (Ocean Nutrition™, Great Salt Lake), separating 300 nauplii for each experimental treatment.

Variation of survival and sexual traits has been demonstrated for different *A. franciscana* populations along time (Vanhaecke et al. 1984; Amat et al. 2007). The *A. franciscana* cysts used came from the Great Salt Lake, a commonly available source for Portuguese aquaculture and aquaria. Other *A. franciscana* strains could have been considered. Deliberate *Artemia* introductions by salt-makers for the improvement of salt production have been described (Amat et al., 2007), and we could have sampled *A. franciscana* from population already established in Portuguese salines. There are however several *A. franciscana* populations in Portugal (Pinto et al., 2012) from which to choose, so that we decided to use a common commercial strain.

2.2. Experimental setup

Survival of the different strains of *Artemia* was assessed throughout their pre-reproductive period under different salinity, temperature, light and food conditions. There were 9 experimental treatments (Table 1) for *A. parthenogenetica* from Aveiro, for *A. parthenogenetica* from Rio Maior and for *A. franciscana*. Each treatment consisted of 10 replicates of 100-ml flasks with 30 *Artemia* each, totalling 2700 animals. Under favourable conditions, the pre-reproductive period ends after about 20 to 25 days (Amat et al., 2007). Experiments therefore took place until 25th day after eclosion, although, under stressing conditions, many animals did not reach maturity by then. Mortality was recorded every two days, at which time the medium of exposure was renewed in each replicate.

We considered 70ppt salinity, 24°C water temperature, 12:12h L:D photoperiod, $\pm 300\,000$ cells ml⁻¹ *Tetraselmis suecica* food supply and aeration at atmospheric pressure (i.e. open containers) as base conditions. In each experiment only one of these parameters was varied to assess its effect on survival; the remaining parameters were kept constant. Salinities of 70ppt, 110ppt and 150ppt were prepared using natural sea water and Tropic Marin Sea Salt® and confirmed with a refractometer. Experimental temperatures of 24°C, 29°C and 34°C $\pm 1^\circ\text{C}$ were maintained keeping the flasks in water baths, with temperatures regularly checked with a thermometer. Photoperiods used were: 12:12h L:D, constant light and constant darkness. Three levels of food supply were prepared, with $\pm 300\,000$ cells ml⁻¹, $\pm 150\,000$ cells ml⁻¹ and $\pm 37\,500$ cells ml⁻¹ of *Tetraselmis suecica* per millilitre of medium. A Neubauer counting chamber was used to count *T. suecica* cells and

accomplish the dilutions necessary to obtain the required densities. Notice that food supply was independent on the number of surviving individuals. This implies a larger individual food supply with ongoing experiment and increasing mortality.

2.3. Data analysis

To analyse our right censored survival data (some individuals survive the experimental period), Kaplan Meier curves with (point-wise) 95% Wald confidence intervals were computed and plotted (Klein and Melvin, 2003; Lumney, 2007). Survival distributions were subsequently compared using the Peto & Peto modification of the Gehan-Wilcoxon test (Harrington and Fleming, 1982). This test is more powerful than the log-rank test when the hazard functions are not parallel and where there is little censoring. It has low power when censoring is high and results can be misleading when a large fraction of subjects are censored at early time points, which is not the case in our data. The Gehan-Wilcoxon test gives more weight to deaths at early time points, so that short-term effects are more important for the discrimination between groups than long-term effects. We tested the null hypothesis that the survival curves were identical: between replicates within each treatment (i.e. for one *Artemia* source and a single set of experimental conditions), between treatment levels applied to one *Artemia* source, and between *Artemia* from different sources considering the same treatment. Subsequent pair-wise tests, using Bonferroni corrected significance thresholds, were used to establish which treatment levels or *Artemia* sources differed. All analyses and plotting were done using the R survival and spline package (Therneau, 2012).

3. Results

Except for the 110ppt and complete darkness treatments and according to the Gehan-Wilcoxon test results, treatment replicates showed no significant differences between them. The 10 replicates of each experimental treatment were pooled to assess differences between *Artemia* sources and between treatment levels. With few exceptions, survivals generally decreased with increasing temperature and salinity, and with decreasing food supply (Figures 2 and 3).

3.1. Comparison between *Artemia* sources

Kaplan-Meier survival curves with respective 95% confidence bounds are presented in Figure 2, with annotations of the Gehan-Wilcoxon test results. Most comparisons between *Artemia* sources showed significant differences. AV and AF had significant survival rates for all experimental treatments. AV and RM were only similar in terms of high temperatures, and RM and AF were similar for low food supply.

Under favourable conditions, i.e. the base treatment, RM was the strain with the highest and AF the strain with the lowest survival rate. AV and RM showed little differences in mortality in the beginning of the experiment, but diverged with time. In terms of temperature, AF was clearly the most resistant to elevated temperatures (29°C and 34°C), followed by AV (at least at 29°C) and RM. Both AV and RM did not survive at 34°C. Considering different salinities, AF had lower survival than RM at 70 ppt and 150 ppt, but higher survival at 110 ppt. AV was the least resistant to high salinities. With half of the food supply (150 000 cells ml⁻¹), AV survived better than the

other two strains, whereas extreme shortage of food ($37\,500\text{ cells ml}^{-1}$) caused high mortality in AV, followed by AF and RM. RM seemed more resistant to food shortage in the later part of the experiment. AV was the least affected by changes in the photoperiod. Both complete darkness and continuous light favoured AV survival in comparison to that of RM and AF. The latter strains reacted with particularly high mortalities to continued, complete darkness.

3.2. Comparison of different treatments for the same *Artemia* strain

Kaplan-Meier survival curves with respective 95% confidence bounds are presented in Figure 3, with annotations of the Gehan-Wilcoxon test results. Comparisons between treatment levels of nearly all variables showed significant differences for the AV and RM *Artemia* strains. AV, on the other hand, was particularly indifferent to food concentration.

The survival of *A. parthenogenetica* from Aveiro decreased significantly with increasing salinity and with increasing temperature; this strain did not tolerate salinities above 110 ppt and a temperature of 34°C . Survival was similar with halved food concentration, yet significantly lower with extreme food shortage ($37\,500\text{ cells ml}^{-1}$). In terms of photoperiod, AV showed significantly higher survival in continuous light and complete darkness conditions.

A. parthenogenetica from Rio Maior showed similar survival patterns, with decreasing survival at higher salinities and temperatures, and with less food supply. However, this strain showed no significant difference between the 100 ppt and 150 ppt salinities and hardly survived the 29°C temperature treatment. RM preferred

the 12:12h photoperiod, followed by continuous light, displaying high mortalities after longer periods in complete darkness.

The survival of *A. franciscana* also decreased significantly with increasing experimental salinity and increasing temperature. The difference between 24°C and 34°C was not significant (notice the Gehan-Wilcoxon test gives more weight to early deaths), but the crossing Kaplan-Meier curves suggest higher long-term mortalities at 34°C. A reduction of the food supply had no significant effect, though Kaplan-Meier estimates suggest increased long-term mortality with extreme food shortage. Survival of AF was favoured by continuous light conditions, although, long-term survival was similar for continuous light and 12:12h L:D and less in complete darkness.

4. Discussion

4.1. Differences between the two *A. parthenogenetica* populations

Results confirm the existence of high variability in the survival of *A. parthenogenetica* diploid strains (Browne, 1992), as both of the analysed strains reacted differently to salinity, temperature, food supply and light conditions. These differences are likely related to locally different selective pressures and consequent (possibly genetic) adaptations (Persoone and Sorgeloos, 1980; Vanhaecke et al., 1984) caused by the very different brine compositions and abiotic conditions.

Compared to *A. parthenogenetica* from Rio Maior, medium to long-term survival rates for *Artemia* from Aveiro were higher 29°C but lower at 24°C. This suggests a possible adaptation of the AV strain to higher temperatures. The ponds in

Aveiro are shallower than those in Rio Maior, and are therefore more likely to reach high water temperatures. A time-series of water temperature measurements at both locations would however be needed to confirm this hypothesis.

A. parthenogenetica from Rio Maior was, on the other hand, more resistant to high salinities (although suffering high mortality) and to severe food shortage. This may also be an adaptation to local conditions, particularly to the characteristics of the supply water. The saline at Rio Maior is a rock salt saline. Naturally dissolved brine is supplied to the ponds from wells where the pumped-up water has a salinity of nearly 150 ppt (personal communication saline technician). Salines in Aveiro are supplied by sea water, sometimes diluted by freshwater from the river Vouga that enters the Ria de Aveiro lagoon at Aveiro. The salinity of the supply water thus never exceeds 35ppt (Vieira and Bio, 2011). Observations of the *Artemia* distribution in the Rio Maior saline, made *in situ*, showed high *Artemia* densities in wing tanks that serve as water storage, where water has much lower salinities than the water in the sampled ponds, probably by blend of rain waters. This would suggest that lower salinities are more favourable to RM *Artemia* survival. This evidence and the observed mortalities at 110ppt and 150ppt lead us to conclude that this population has difficulties in keeping high numbers of individuals alive at salinities of 150ppt, such as those found in the salina supply water, and that the wing tanks allow the maintenance of large amounts of *Artemia* during the whole year.

In terms of food availability, the Aveiro saline complex has a greater diversity of species that are part of the *Artemia* diet (Vieira and Bio, 2011). Being part of the Ria de Aveiro estuary, its salines have also higher levels of fertilization through considerable

amounts of dissolved nutrients of anthropogenic and natural (e.g. waterbird droppings) origins (Lopes et al., 2007). In contrast, the rock salt brine at Rio Maior has little nutrient input and biodiversity, due to the characteristics of its water source and its inland location (Calado and Brandão, 2009). This may explain the greater resistance (adaptation) of *Artemia* from RM to severe food shortage.

We observed that *A. parthenogenetica* from Rio Maior was more sensitive to extreme photoperiods (complete darkness or complete light) than that from Aveiro. This is difficult to explain, but may suggest an overall better light tolerance for the Aveiro strain. In Aveiro, saline ponds reach lower depths and are thus exposed to high radiation and temperature. Aveiro lays also more to the North, causing longer days in summer and longer nights in winter; though that difference should be too little to explain the different survival rates found. There is little information in literature about the effect of photoperiods on *Artemia*, as most experiments use the 12:12h L:D setup.

4.2. Differences between *A. parthenogenetica* populations and *A. franciscana*

The main difference between the studied Portuguese *A. parthenogenetica* strains and *A. franciscana* is the extreme tolerance of *A. franciscana* to high water temperatures. This species had a much higher survival at 29°C, and some individuals even survived 34°C, a temperature that was lethal for the parthenogenetic strains. Results suggest that the optimum temperature for survival of *A. franciscana* is probably closer to 29°C than 24°C, since the observed survival was significantly higher for 29°C than for 24°C. These results confirm the ability of *Artemia franciscana* to tolerate a large range of temperatures found by several authors (Wear and Haslett,

1986; Browne, 1988; Browne et al., 1991). Browne and Wanigasekera (2000) consider *Artemia franciscana* to be a euryhaline and eurythermal species. Regarding our experimental salinities, *A. franciscana* was clearly more adapted to intermediate/high salinities. It was the only *Artemia* source with a considerable proportion of live individuals after 25 days at a salinity of 110ppt, showing a better adaptation to this salinity than the Portuguese parthenogenetic strains. At the highest experimental salinity however nearly no *A. franciscana* survived. Only the RM strain showed resistance to this salinity, as could be expected given the hypersaline brine it lives in.

Contrary to *A. parthenogenetica* from Aveiro, *A. franciscana* and *A. parthenogenetica* from Rio Maior survive, to some extent, severe food shortage. This resistance is particularly critical for the Aveiro salina, where *A. franciscana* would gain advantage over the native *A. parthenogenetica* and invasive ability if food supply became scarce.

Analysing the impact of different photoperiods on the studied strains, we found that *A. franciscana* does not tolerate total darkness well, just as the RM strain. This is interesting because this factor has rarely been assessed, but it can hardly be linked to the invasiveness of the species. Little can be inferred from the behaviour in the wild where this situation does practically not occur. All strains studied showed good survival rates in total light and 12 hours light 12 hours darkness photoperiod.

Considering the variables and treatment levels studied, *A. franciscana* was rarely at a disadvantage in comparison to the native parthenogenetic strains. Compared to *A. parthenogenetica* from Aveiro, its survival was only worse when exposed to extreme photoperiod conditions. Compared to the parthenogenetic strain

from Rio Maior, *A. franciscana* was only more vulnerable at the lowest studied temperature (24°C), at very high salinities (150 ppt) and (in the long term) at very low food concentrations (37 500 cells ml⁻¹). These conditions are typical of the saline environment found in Rio Maior, suggesting that the biotope characteristics constitute a limiting factor to the invasion by *A. franciscana*. There is however at least one other factor to consider: the geographical inland location of the Rio Maior salines, which lies far from the main bird migration routes, far from fish farming facilities and urban areas with aquaria, reducing the possibility of an accidental introduction (Amat et al. 2007).

There are no records of *A. franciscana* observed in the Troncalhada salina (Aveiro), although this invasive species has been recorded in other salines of the same complex, not too distant from the studied one (Amat et al, 2007), and the saline is inhabited by numerous bird species, which could be an introduction vector for the invasive *Artemia* strain. This fact, added to the fact that the parthenogenetic strain from Aveiro performed worse than *A. franciscana* for almost all of our studied factors and treatment levels, suggests that the maintenance of the native parthenogenetic strain and absence of *A. franciscana* in the Troncalhada saline may be due to other strain-specific traits, next to survival, or to other local biotope-specific factors. Further studies are necessary to test other traits related to population dynamics, which determine biological fitness and life span, such as the time of pre-reproductive and reproductive periods (Allan, 1976), the type of reproduction, as well as different species concurrence and crowding, (Barata et al., 1995, 1996a,b; Browne et al., 1984, 1988, 1991). The location of Troncalhada saline may also hold the key to understand the lack of invasion. This saline is located at the inland limit of the Ria de Aveiro,

immediately next to the city of Aveiro. It is an urbanized saline in terms of water supply, being fed by an urban channel with often highly polluted water, including high levels of heavy metals and pesticides (e.g. Martins et al, 2010). A possible explanation for the persistence of the local native strain may thus be related to its greater ability to tolerate and survive the different contaminants polluting this saline. In addition to population dynamics variables, the presence of a chemical barrier in the environment is a factor that should be considered in future studies to determine the reasons for a greater or lesser success of *Artemia* species at extinction risk, which continue living in certain biotopes.

5. Conclusions

The parthenogenetic *Artemia* strains found in Portugal are vulnerable, and protective measures are needed to avoid the introduction of exotic *Artemia* species in the few locations where native species persist. These studied Portuguese parthenogenetic populations showed great variability in their physiological response when exposed to different abiotic conditions, which is in agreement with results found for other *A. parthenogenetica* populations. The *A. parthenogenetica* strain from Rio Maior was more resistant to conditions that are similar to its local habitat than *A. franciscana*. This and the inland location of the Rio Maior saline may explain why this saline has not yet been invaded by *A. franciscana*. On the other hand, *A. franciscana* outcompeted the parthenogenetic strain from Aveiro for nearly all of the different studied factors. There have to be other factors preventing invasion by *A. franciscana*, possibly related to unstudied population traits and/or to an adaptive tolerance to

pollution (e.g. heavy metals) of the local native strain. New investigations on chemical conditions and tolerances, especially those related to pollution, are hence needed for a better understanding of the permanence of this and other parthenogenetic strains in European salines.

This survival study will shortly be complemented with a study of the reproductive fitness of these *Artemia* strains. Life table parameters obtained under different experimental conditions will allow insight into the conditions' effects on the reproduction, next to the survival, and help us to design a model for the distribution of *A. franciscana* in the Iberian and Mediterranean region.

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521 **Table 1.** Experimental treatment conditions; each treatment was applied to 10 replicates with
 522 30 animals each. Treatments used to make comparisons per treatment condition are marked
 523 in bold.

Treatment	Salinity (ppt)	Temperature (°C)	Light (h)	Food supply (cells ml ⁻¹)
1	70	24	12	300000
2	110	24	12	300000
3	150	24	12	300000
4	70	24	12	300000
5	70	29	12	300000
6	70	34	12	300000
7	70	24	12	150000
8	70	24	12	37500
9	70	24	0	300000
10	70	24	24	300000

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Figure 1. Location of the Troncalhada salina near Aveiro (A) and the salina near Rio Maior (B)

Figure 2. Kaplan–Meier survival estimates with 95% confidence bounds for *Artemia parthenogenetica* (A. part.) from Aveiro and Rio Maior and for *A. franciscana* under different culture conditions. Different letters indicate significant ($\alpha = 0.05$) differences between *Artemia* sources, according to the Gehan–Wilcoxon test results (Bonferroni adjusted for multiple comparisons).

Figure 2. Continuation.

Figure 3. Kaplan–Meier survival estimates with 95% confidence bounds for *Artemia parthenogenetica* from Aveiro and Rio Maior and for *A. franciscana* depending on culture conditions. Different letters indicate significant ($\alpha= 0.05$) differences between treatment levels, according to the Gehan-Wilcoxon test results (Bonferroni adjusted for multiple comparisons).

Figure 3. Continuation.