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ASPECTOS RELEVANTES EN LA CONSERVACIÓN DE ANFIBIOS EN LA REGIÓN DE MURCIA:

EFECTOS DE LA CONTAMINACIÓN POR FERTILIZANTES SOBRE *Pelophylax perezi* (SEOANE, 1885).

RELEVANT ASPECTS IN AMPHIBIAN CONSERVATION IN THE PROVINCE OF MURCIA:
EFFECTS OF FERTILIZER POLLUTION ON *Pelophylax perezi* (SEOANE, 1885).

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Aspectos relevantes en la conservación de anfibios en la Región de Murcia: efectos de la contaminación por fertilizantes sobre *Pelophylax perezi* (Seoane, 1885)

Memoria presentada para optar al grado de Doctor en Biología por el Licenciado en Biología

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A mi familia y, muy especialmente, a mis padres, Paco y María

With magic, you can turn a frog into a prince. With science, you can turn a frog into a Ph.D. and you still have the frog you started with.

Terry Pratchett, Ian Stewart & Jack Cohen. 2002. *The Science of Discworld*. Ebury Press, Londres.

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La realización de un proyecto de investigación, con independencia de sus objetivos, es una tarea compleja, por gratificante que pueda revelarse. Los numerosos obstáculos, conspicuos o sutiles, que aparecen durante el desarrollo de una investigación sólo pueden ser salvados gracias a la contribución de un ejército de personas, y mi caso no es una excepción. Así, debo agradecer en primer lugar a mis directores, Miguel Tejedo y Mar Torralva, su apoyo logístico e, indudablemente, intelectual. Las discusiones sobre las cuestiones planteadas directamente en la presente Tesis Doctoral, así como sobre otras más indirectas, pero no por ello menos interesantes, han resultado enormemente estimulantes para mí. Sin duda alguna, durante este periplo predoctoral he aprendido mucho de ellos.

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GENERAL ABSTRACT (English version)

Current extinction rates due to the direct and indirect consequences of human intervention on natural systems are several orders of magnitude higher than those recorded throughout the historical evolution of the Earth. Consequently, minimizing the loss of global biodiversity has arisen as one of the major challenges to face nowadays. Amphibians are the most threatened group of vertebrates. The decline and local extinction of amphibian populations and species has been observed in several regions of the world. Although such a decline may be attributed to natural demographic fluctuations, evidence supporting the hypothesis of global amphibian decline due to the direct and indirect action of humankind has been reported. Six factors have been hypothesized as possible causes for the observed decline. These factors, which may act at either global or local scale, have been classified into two different classes, according to either the understanding of their effects on amphibians or the time these vertebrates have been exposed to them. Thus, class I hypotheses would include those factors that have been acting for at least the last 100 years (exotic species, habitat destruction and alteration and overexploitation), whereas class II hypotheses would comprise those factors for which there is a poor understanding and which are affecting amphibians from more recent times (global change, emergent diseases and chemicals). Moreover, the effects of the interaction among several stressors have recently become of great concern among researchers because its additive or synergistic effects may exacerbate amphibian vulnerability to extinction. However, in spite of the information existing on the impacts of the stressing factors mentioned, its knowledge is far from being exhaustive, and further studies are necessary to complete the existing database on their effects.

Although chemicals are cited as one of the major causes to explain the decline of amphibian populations, most studies dealing with the impact of these pollutants have mainly focused on the effect of heavy metals, pesticides, polychlorinated biphenyls and polycyclic aromatic hydrocarbons. Nevertheless, other types of pollutants, such as road de-icers or pharmaceuticals degradates, may also produce a negative impact on fitness-related traits. Among this last group of pollutants, the interest to accurately assess the effects of fertilizers and other nitrogenous compounds is increasing, since these compounds are distributed worldwide in natural habitats and their presence is expected to increase in the future. Moreover, because nitrogen pollution has been described to produce both lethal and sublethal effects on amphibians, improving the understanding of the real effects that the exposure to nitrogenous compounds may produce should be a priority. Consequently, the amount of studies devoted to assess such effects is raising. However, to date, two main shortcomings in the analyses of nitrogenous pollution as a threat to amphibians exist: first, most of the studies have been carried out in laboratory conditions. Although this is an essential starting point, it is necessary to perform further studies to assess whether the results obtained in the laboratory ensue in more natural conditions. Second, data on the potential adaptive response of amphibian populations to nitrogenous pollutants are still lacking and only a few reports on the inter and intra-specific variation regarding the tolerance to nitrogenous compounds have been published. Therefore, this underlines the need to perform further researche to accurately determine the real impact of nitrogenous pollution on amphibians.

In this context, the present PhD thesis looks at the impact of nitrogenous compounds on the Iberian water frog, *Pelophylax perezi* (Seoane, 1885), with the following objectives: 1) Larvae and metamorphs of *P. perezi* were exposed to ammonium chloride (NH_4Cl), sodium nitrite ($NaNO_2$) and sodium nitrate ($NaNO_3$) to

determine: a) larval tolerance limits and habitat selection of postmetamorphic individuals in laboratory conditions, b) larval survival and sublethal effects in different venues, and, c) larval survival and sublethal effects when pollutants were presented either isolated or combined in different venues. 2) The existence of interpopulational divergences in larval survival and sublethal effects due to nitrogenous pollution and the possible evolution of local adaptation. 3) To provide an overview of pollution effects on amphibians during their aquatic and metamorphic stages by conducting a meta-analysis.

To examine the tolerance limits of the studied species, the effects of the acute exposure to nitrogenous compounds on larval survival was studied in the laboratory for 72 h (in the case of nitrite and nitrate) or 96 h (for ammonium). To analyse the effect of nitrogenous pollution, larvae were exposed for 21 days to sublethal concentrations of ammonium, nitrite and nitrate and to some of their combinations in the laboratory, and to sublethal concentrations of ammonium, isolated or combined with nitrite and nitrate, in mesocosms. Moreover, in a field experiment, enclosed larvae were exposed for 21 days to natural streams showing differing levels of nitrogenous pollution. For all these experiments, the response variables studied included survival, final mass, growth, morphological traits or behavioral endoints (i.e. number of censuses in which larvae were observed inactive or at the bottom of the experimental beakers, food consumption and escape ability). The hypothesis suggesting that the presence in the water column of ammonium, either isolated or combined with nitrite and nitrate, would affect the use of aquatic habitats by metamorphs was explored in laboratory conditions for 14 days.

The existence of interpopulational divergences in the tolerance to sublethal nitrogenous pollution was explored in the laboratory by comparing survival and larval performance of two populations inhabiting polluted habitats with two populations located in less polluted environments. Additionally, the hypothesis of local adaptation

was examined both in mesocoms and field-based enclosures by considering larvae from one polluted population and from two less polluted ones.

The results obtained for the acute exposure to ammonium, nitrite and nitrate in the laboratory showed that larval mortality increased with increasing concentrations of these ions, as well as with raising exposure times. Thus, the negative impact of acute nitrogenous pollution corresponds to its chronic effect rather than to the initial exposure. Mean lethal concentration values (LC_{50}) for ammonium, nitrite and nitrate decreased as time passed. For ammonium, lower lethal concentration values to that concentrations recorded in the field were estimated after a period of 96 h, suggesting that ammonium pollution may be a threat to *P. perezi* populations.

As regards the analysis of the effect of sublethal nitrogenous pollution, the exposure to high concentration of ammonium, nitrite and nitrate, either isolated or combined, for 21 days significantly increased larval mortality and reduced food consumption in laboratory conditions, existing evidence for the significant reduction of larval mass at higher ammonium and nitrite concentrations. Moreover, the number of censuses that larvae were detected inactive or at the bottom of the experimental beakers was reduced by the exposure to lower concentrations of ammonium (only in the case of inactivity) or nitrate or by the combination of low nitrite concentration with nitrate and ammonium.

The analysis of habitat selection of metamorphic individuals revealed that, when they were exposed in laboratory conditions to ammonium acting isolated or combined with nitrite and nitrate, the presence of nitrogenous ions in the water column did not involve the avoidance of aquatic environments, although significant inter-individual variation in pollution avoidance was detected.

In addition to the experiments carried out in laboratory conditions, the effects of nitrogenous pollution were explored in more natural conditions. Thus, in outdoor mesocosms, the exposure to sublethal concentrations of ammonium, isolated or combined with nitrite and nitrate, did not affect larval survival after a period of 21 days, although it significantly reduced body and tail depth, final mass and growth. A different pattern was found for field enclosures. Larval mortality increased when larvae of the studied species were exposed for 21 days to natural permanent streams containing a high degree of nitrogenous pollution, although surviving larvae showed higher values for morphological traits, final mass and growth than those tadpoles exposed to less polluted streams. Treatments or field localities did not affect either distance swum nor swimming speed. Nevertheless, the influence of morphological traits on swimming performance greatly varied across populations of origin and treatments. A positive trade-off between growth and swimming speed was detected in the case of the exposure to high concentration of ammonium acting alone and for the exposure to polluted habitats.

The exposure to the combination of sublethal concentrations of ammonium, nitrite or nitrate was more harmful than the effect of these ions acting isolated in both laboratory and mesocosms experiments. When experiments were run in the laboratory this negative effect may fit well to a synergistic effect in the case of larval survival, final mass and food consumption, whereas it was additive for behavioural traits (i.e. number of censuses tadpoles were found inactive or at the bottom of the experimental units).

As regards the analysis of the interpopulational variation in the tolerance to nitrogenous pollution, population-specific responses to treatments were detected in both laboratory and mesocosm experiments, suggesting local adaptation to polluted environments, since larvae from populations inhabiting highly polluted environments

showed higher larval survival in the face of treatments enriched with high nitrogen concentration than those from populations coming from less polluted localities. Nevertheles, when larvae were exposed to natural field sites, no population divergence in tolerance to pollution was detected.

The overall impact of chemical pollutants on amphibians as a group was explored by meta-analysis. This statistical procedure allows to analyse data obtained in independent studies. Unlike traditional reviews based on vote-counting methods, meta-analysis techniques show high statistical power and provide a reliable way of determining the overall magnitude of the impact of pollutants on amphibians, as well as a way of comparing the effects among categories for *a priori* defined groups. To run meta-analyses, a thorough review of the publications dealing with the impact of pollutants on amphibians was carried out. Morevoer, several authors kindly provided some of their unpublished databases. The studies obtained were analysed and selected whether the concentrations used in the experiments were ecologically relevant for the location; mean and standard deviation values for both a control and an experimental group, as well as the number of cases used to compute these statistics, were clearly provided; the effect of only one type of pollutant was studied and data for amphibian survival, time to hatching, time to metamorphosis, total length, weight or abnormality rate were reported. Moreover, to assess the effect and magnitude of the interaction between pollutants and other biotic and abiotic factors on amphibian survival, studies fitting the above criteria and showing a factorial design were selected. Mean and standard deviation values for both a control and an experimental group, as well as the number of cases used to compute these statistics, were recorded from each publication or unpublished database selected for inclusion in the meta-analyses to assess the pollutant effect size. The results obtained showed that pollution is an important threat to

amphibians, negatively affecting survival, size and development. However, time to hatching and time to metamorphosis were unaffected by chemical pollutants. In spite of the overall impact observed for most of the studied variables, wide variation regarding experimental venue, developmental stage at the beginning of the experiment and type of pollutant was detected. Significant differences among amphibian families were only detected for time to hatching, the impact of chemical pollution being higher for ambystomatid salamanders. In this case, significant phylogenetic autocorrelation was detected. Moreover, the exposure to a wide range of biotic and biotic stressing factors affected survival more severely than the exposure to full pollutant stressors, no evidence existing for a significant interaction between different types of stressors.

In conclusion, the results obtained support the hypothesis describing chemical pollution as a major threat to amphibians. Both the experimental studies carried out on the effect of nitrogenous pollution on *P. perezi* and the meta-analytic review show that the exposure to both lethal and sublethal concentrations of pollutants may affect larval survival and other sublethal endpoints. Although it is difficult to infer the response of a population from the effects on inviduals, these effects indicate that the negative impact of nitrogenous pollution may play an important role in the decline of amphibian populations. However, local adaptation to polluted environments is an alternative to extinction and, consequently, chemical pollution in general and, more specifically, nitrogenous compounds may be an important factor directing evolution in amphibians, as the results obtained suggest. Additionally, the different results obtained for the different experiments carried out in laboratory, mesocosm or enclosure conditions, together with the conclusion derived from the meta-analysis, point to the great relevance of the venue when assessing the impact of a stressor on amphibians.

RESUMEN GENERAL (versión española)

Las tasas de extinción actuales debidas a las consecuencias directas e indirectas de la intervención humana sobre los sistemas naturales son varios órdenes de magnitud superiores a aquéllas registradas durante la evolución histórica de la Tierra. Como consecuencia, uno de los principales desafíos a afrontar actualmente consiste en la minimización de la pérdida global de biodiversidad. Los anfibios son el grupo de vertebrados más amenazados. Se ha observado el declive y las extinciones locales tanto de poblaciones como de especies de anfibios en varias regiones del mundo. Aunque este declive puede ser atribuido a fluctuaciones demográficas naturales, se han descrito evidencias que apoyan la hipótesis del declive global de los anfibios debido a la acción directa e indirecta del hombre. Se han señalado seis factores como causas eventuales de los declives observados. Estos factores, los cuales pueden actuar tanto a escala global como local, han sido clasificados en dos clases diferentes, en función tanto de la comprensión de sus efectos sobre los anfibios como del tiempo que estos vertebrados han estado expuestos a ellos. Así, las hipótesis clase I incluirían aquellos factores que han estado actuando al menos durante los últimos 100 años (especies exóticas, alteración y destrucción del hábitat y sobreexplotación) mientras que las hipótesis clase II comprenderían aquellos factores para los que existe un conocimiento pobre de sus efectos y los cuales están afectando a los anfibios desde épocas más recientes (cambio climáticos, enfermedades emergentes y sustancias químicas). Además, el efecto de la interacción entre varios agentes estresantes ha despertado recientemente un gran interés entre los investigadores dado que sus efectos aditivos o sinérgicos pueden exacerbar la vulnerabilidad de los anfibios a la extinción. Sin embargo, a pesar de la información disponible sobre los impactos de los factores estresantes mencionados, su conocimiento

está lejos de ser exhaustivo, por lo que son necesarios más estudios para completar la base de datos existente sobre sus efectos.

Aunque las sustancias químicas son una de las causas principales que se han argumentado para explicar el declive de las poblaciones de anfibios, la mayor parte de los estudios relacionados con el impacto de estos contaminantes estudian el efecto de metales pesados, pesticidas, bifenilos policlorados e hidrocarburos aromáticos policíclicos. Sin embargo, otros tipos de contaminantes, como sales descongelantes o sustancias derivadas de la degradación de productos farmacéuticos, pueden producir un impacto negativo sobre caracteres relacionados con el estado físico de los individuos. Entre este último grupo de contaminantes, está aumentando el interés por establecer adecuadamente los efectos de fertilizantes y otros compuestos nitrogenados, ya que estos compuestos están distribuidos por todo el mundo en hábitats naturales y se espera que su presencia se incremente en el futuro. Además, dado que se ha descrito que la contaminación nitrogenada produce tanto efectos letales como subletales sobre los anfibios, sería de gran interés mejorar la comprensión de los efectos reales que la exposición a compuestos nitrogenados puede producir. Como consecuencia, se está incrementando la cantidad de estudios dedicados a establecer estos efectos. Sin embargo, hasta la fecha existen dos defectos principales en el análisis de la contaminación nitrogenada como amenaza para los anfibios: en primer lugar, la mayor parte de los estudios se han realizado en condiciones de laboratorio. Aunque éste es un punto de partida esencial, es necesario realizar más estudios para establecer si los resultados obtenidos en el laboratorio se obtienen también en condiciones más naturales. En segundo lugar, faltan datos sobre la potencial respuesta adaptiva de las poblaciones de anfibios a los contaminantes nitrogenados y sólo se han descrito unas pocas evidencias sobre la variación inter e intra-específica en relación a la tolerancia a

compuestos nitrogenados. Así, este hecho señala la necesidad de realizar más estudios para determinar adecuadamente el impacto real de la contaminación nitrogenada en los anfibios.

En este contexto, se estudió en la presente Tesis Doctoral el impacto de compuestos nitrogenados sobre la rana común, *Pelophylax perezi* (Seoane, 1885), con los siguientes objetivos: 1) Se expusieron larvas y metamórfícos de *P. perezi* a cloruro amónico (NH_4Cl), nitrito sódico ($NaNO_2$) y nitrato sódico ($NaNO_3$) para determinar: a) los límites de tolerancia larvaria y la selección de hábitat de individuos postmetamórficos en condiciones de laboratorio, b) la supervivencia larvaria y los efectos subletales en diferentes condiciones experimentales, y, c) la supervivencia larvaria y los efectos subletales cuando los contaminantes se presentaron tanto aislados como combinados en diferentes condiciones experimentales. 2) Estudiar la existencia de divergencias interpoblacionales en la supervivencia larvaria y en los efectos subletales de la contaminación nitrogenada, y la posible evolución de adaptación local. 3) Proporcionar una visión general de los efectos de la contaminación sobre los anfibios durante sus estadios acuáticos y metamórficos mediante la realización de un meta-análisis.

Para examinar los límites de la tolerancia de la especie estudiada, se estudiaron los efectos de la exposición aguda a compuestos nitrogenados sobre la supervivencia larvaria en el laboratorio durante 72 h (en el caso de nitrito y nitrato) o 96 h (para amonio). Para analizar el efecto de la contaminación nitrogenada, se expusieron larvas durante 21 días a concentraciones subletales de amonio, nitrito y nitrato y a algunas de sus combinaciones en el laboratorio, y a concentraciones subletales de amonio, aislado o combinado con nitrito y nitrato, en mesocosmos. Además, en un experimento de campo, se expusieron larvas enjauladas durante 21 días a arroyos naturales que muestran

diferentes niveles de contaminación nitrogenada. Para todos estos experimentos, las variables de respuesta estudiadas incluyeron supervivencia, masa final, crecimiento, rasgos morfológicos o parámetros etológicos (i.e. número de veces que las larvas se detectaron inactivas o sobre el fondo de los contenedores experimentales, consumo de alimento y habilidad de huida). Finalmente, se examinó en condiciones de laboratorio durante 14 días la hipótesis que sugiere que la presencia en la columna de agua de amonio, tanto aislado como combinado con nitrito y nitrato, afectaría el uso de los hábitats acuáticos por los metamórficos.

Se exploró la existencia de divergencias interpoblacionales en la tolerancia a la contaminación nitrogenada subletal en el laboratorio mediante la comparación de la supervivencia y la respuesta larvaria de dos poblaciones que habitan hábitats contaminados con dos poblaciones localizadas en ambientes menos contaminados. Adicionalmente, se examinó la hipótesis de la adaptación local tanto en mesocosmos como en corrales localizados en el campo mediante la consideración de larvas procedentes de una población contaminada y de otras dos menos contaminadas.

Los resultados obtenidos para la exposición aguda de amonio, nitrito y nitrato en el laboratorio mostraron que la mortalidad larvaria aumentó con el incremento de las concentraciones y del tiempo de exposición a estos iones. Así, el impacto negativo de la contaminación nitrogenada aguda corresponde a su efecto crónico, en lugar de a la exposición inicial. Los valores correspondientes a la concentración letal media (LC_{50}) obtenidos para amonio, nitrito y nitrato disminuyeron con el tiempo. Para amonio, se estimaron concentraciones letales inferiores a las concentraciones registradas en el campo tras un periodo de de 96 h, lo que sugiere que la contaminación por amonio puede ser una amenaza para las poblaciones de *P. perezi*.

En relación al análisis del efecto de la contaminación nitrogenada subletal, la exposición a altas concentraciones de amonio, nitrito y nitrato, aislados o combinados, durante 21 días incrementó significativamente la mortalidad larvaria y redujo el consumo de alimento en condiciones de laboratorio, existiendo evidencias de la significativa reducción de la masa larvaria a altas concentraciones de amonio y nitrito. Además, se redujo el número de veces que las larvas se detectaron inactivas o sobre el fondo de los contenedores experimentales debido a la exposición a bajas concentraciones de amonio (sólo en el caso de la inactividad) o nitrato, o por la combinación de baja concentración de nitrito con nitrato y amonio.

El análisis de la selección de hábitat de individuos metamórficos reveló que, al ser expuestos en condiciones de laboratorio a amonio actuando aislado o combinado con nitrito y nitrato, la presencia de iones nitrogenados en la columna de agua no implicó la evitación del medio acuático, aunque se detectó una variación inter-individual significativa.

Adicionalmente a los experimentos realizados en condiciones de laboratorio, se exploraron los efectos de la contaminación nitrogenada en condiciones más naturales. Así, la exposición a concentraciones subletales de amonio, aislado o combinado con nitrito y nitrato, no afectó a la supervivencia larvaria tras un periodo de 21 días en mesocosmos instalados al aire libre, aunque redujo significativamente la altura del cuerpo y de la cola, la masa final y el crecimiento. Sin embargo, se encontró un patrón diferente para los corrales de campo. Se incrementó la mortalidad larvaria cuando las larvas de la especie estudiada se expusieron durante 21 días a arroyos permanentes naturales que contienen un grado elevado de contaminación nitrogenada, aunque las larvas supervivientes mostraron valores superiores para los rasgos morfológicos, masa final y crecimiento que aquellos renacuajos expuestos a arroyos menos contaminados.

Los tratamientos o las localidades de campo no afectaron ni a la distancia nadada ni a la velocidad natatoria. Sin embargo, la influencia de los rasgos morfológicos sobre la habilidad natatoria varió en gran medida entre poblaciones de origen y tratamientos. Se detectó una relación positiva entre el crecimiento y la velocidad natatoria en el caso de la exposición a alta concentración de amonio actuando aislado y a hábitats contaminados.

La exposición a la combinación de concentraciones subletales de amonio, nitrito o nitrato fue más dañina que el efecto de estos iones actuando de modo aislado tanto en experimentos de laboratorio como de mesocosmos. Cuando los experimentos se realizaron en el laboratorio, este efecto negativo se ajustó a un efecto sinérgico en el caso de la supervivencia larvaria, masa final y consumo de alimento, mientras que fue aditivo para variables etológicas (i.e. número de veces que los renacuajos se encontraron inactivos o en el fondo de las unidades experimentales).

Respecto al análisis de la variabilidad interpoblacional de la tolerancia a la contaminación nitrogenada, se detectaron respuestas a los tratamientos específicas de la población de origen tanto en los experimentos de laboratorio como de campo, lo que sugiere la existencia de adaptación local a ambientes contaminados, ya que las larvas procedentes de poblaciones que habitan ambientes altamente contaminados mostraron mayor supervivencia larvaria a los tratamientos enriquecidos con alta concentración de nitrógeno que aquéllas procedentes de poblaciones que ocupan localidades menos contaminadas. Sin embargo, cuando las larvas se expusieron a localidades naturales, no se detectó divergencia poblacional en la tolerancia a la contaminación.

El impacto global de los contaminantes químicos sobre los anfibios como grupo se exploró mediante meta-análisis. Esta técnica estadística permite analizar datos obtenidos en estudios independientes. A diferencias de revisiones tradicionales basados en

métodos *vote-counting*, las técnicas meta-analíticas muestran un alto poder estadístico y proporcionan un modo fiable de determinar la magnitud global del impacto de los contaminantes sobre los anfibios, así como de comparar los efectos entre categorías de grupos definidos *a priori*. Para realizar meta-análisis, se realizó una profunda revisión de las publicaciones relacionadas con el impacto de los contaminantes sobre los anfibios. Además, varios autores proporcionaron amablemente algunas de sus bases de datos inéditas. Se analizaron los estudios obtenidos y se seleccionaron aquéllos que cumplieron los siguientes criterios: se usaron concentraciones ecológicamente relevantes para la localidad; se presentaron valores medios y de desviación estándar tanto para un grupo control como para uno experimental, así como el número de casos utilizados para calcular estos estadísticos; se analizó el efecto de un único tipo de contaminante y se presentaron datos para la supervivencia, tiempo hasta la eclosión, tiempo hasta la metamorfosis, longitud total, masa o tasa de malformación. Además, para establecer el efecto y la magnitud de la interacción entre contaminantes y otros factores bióticos y abióticos sobre la supervivencia de los anfibios, se seleccionaron estudios que cumplieron los requisitos anteriores y que mostraron un diseño factorial. Para establecer el tamaño del efecto de los contaminantes, de cada publicación o base de datos inédita seleccionada para su inclusión en el meta-análisis se obtuvieron los valores medios y de desviación estándar para tanto un grupo control como un grupo experimental, así como el número de casos utilizados para calcular estos estadísticos. Los resultados obtenidos evidenciaron que la contaminación es una importante amenaza para los anfibios, al afectar negativamente la supervivencia, talla y desarrollo. Sin embargo, los contaminantes químicos no afectaron ni al tiempo hasta la eclosión y ni al tiempo hasta la metamorfosis. A pesar del impacto general observado para la mayor parte de las variables estudiadas, ha sido detectada una gran variación en relación a las

condiciones experimentales, estadio de desarrollo al inicio del experimento y tipo de contaminante. Se detectaron diferencias significativas entre familias de anfibios para el tiempo hasta la eclosión, siendo el impacto de la contaminación química mayor para las salamandras ambistomátidas. En este caso, se detectó la existencia de autocorrelación filogenética significativa. Además, la exposición a un rango amplio de agentes estresantes bióticos y abióticos afectó más severamente a la supervivencia que la exposición exclusivamente a contaminantes, no existiendo evidencias sobre una interacción significativa entre diferentes tipos de agentes estresantes.

En conclusión, los resultados obtenidos apoyan la hipótesis que describe a la contaminación química como una de las principales amenazas para los anfibios. Tanto los estudios experimentales llevados a cabo sobre el efecto de la contaminación nitrogenada sobre *P. perezi* y la revisión meta-analítica evidencian que la exposición tanto a concentraciones letales como subletales de contaminantes pueden afectar a la supervivencia larvaria y a otras variables subletales. Aunque es difícil inferir la respuesta de una población a partir de los efectos sobre los individuos, estos efectos indican que el impacto negativo de la contaminación nitrogenada puede jugar un papel importante en el declive de las poblaciones de anfibios. Sin embargo, la adaptación local a ambientes contaminados es una alternativa a la extinción y, como consecuencia, la contaminación química en general y, más específicamente, los compuestos nitrogenados pueden ser un importante factor que conduzca la evolución de los anfibios, como sugieren los resultados obtenidos. Adicionalmente, los diferentes resultados obtenidos para los diferentes experimentos realizados en condiciones de laboratorio, mesocosmos o corrales, y la conclusión derivada del meta-análisis, revelaron la gran importancia de las condiciones experimentales al establecer el impacto de un agente estresante sobre los anfibios.

ESTRUCTURA DE LA PRESENTE TESIS DOCTORAL

La finalidad de la presente Tesis Doctoral fue ampliar la información existente sobre los efectos que los compuestos nitrogenados producen en los anfibios, y profundizar en el conocimiento del impacto que los contaminantes químicos tienen, de modo global, sobre este grupo de vertebrados.

Esta Tesis Doctoral se ha estructurado en cuatro grandes bloques, los cuales agrupan tanto los capítulos relacionados con los antecedentes existentes sobre la temática planteada por la Tesis, como con los resultados obtenidos y las principales conclusiones extraídas de los mismos. Cada uno de los bloques mencionados incluye uno o varios capítulos, los cuales, exceptuando los introductorios y el referente a las conclusiones generales, se han organizado atendiendo a las secciones habituales en cualquier publicación científica (Introducción, Material y Métodos, Resultados, Discusión y Referencias). De este modo, a pesar del inevitable inconveniente de la repetición de información en capítulos relacionados, cada uno de ellos es relativamente independiente de los restantes, facilitando la comprensión e interpretación de los resultados presentados.

A continuación se detallan los capítulos comprendidos en cada uno de los bloques que componen la presente Tesis Doctoral.

Bloque I.- Este bloque describe los conocimientos existentes sobre el impacto de diferentes factores de amenaza sobre los anfibios a escala global, estatal (i.e. España) y regional (i.e. Región de Murcia), las principales características de las poblaciones de anfibios en España y en la Región de Murcia, los objetivos planteados en el presente

trabajo y la descripción del área de estudio, de la especie estudiada y de la metodología empleada en la presente Tesis Doctoral. Incluye los siguientes capítulos:

Capítulo 1. *Introducción y Objetivos* (manuscrito inédito).

Capítulo 2. *Área de estudio, descripción de la especie estudiada y sinopsis metodológica* (manuscrito inédito).

Bloque II.- Este bloque presenta los resultados obtenidos tras evaluar el impacto letal y subletal de cloruro amónico, nitrito sódico y nitrato sódico, de manera aislada o combinada, en ejemplares larvarios y juveniles de *Pelophylax perezi* (Seoane, 1885) en condiciones de laboratorio. Incluye los siguientes capítulos:

Capítulo 3. *Estimating mean letal concentrations of three nitrogenous compounds for the Iberian water frog,* Pelophylax perezi *(Seoane, 1885), larvae* (manuscrito publicado bajo la forma: Egea-Serrano, A.; Tejedo, M. & Torralva, M. 2009. Estimating mean lethal concentrations of three nitrogenous compounds for the Iberian waterfrog, *Pelophylax perezi* (Seoane, 1885), larvae. Revista Española de Herpetología, 23: en prensa).

Capítulo 4. *Populational divergence in the impact of three nitrogenous compounds and their combination on larvae of the frog* Pelophylax perezi *(Seoane, 1885)* (manuscrito publicado bajo la forma: Egea-Serrano, A.; Tejedo, M.; Torralva, M. 2009. Populational divergente in the impact of three nitrogenous compounds and their combination on larvae of the frog *Pelophylax perezi* (Seoane, 1885). Chemosphere, 76: 869-877).

Capítulo 5. *Examining the impact of three nitrogenous compounds and their combination on inactivity level and habitat use of larvae of* Pelophylax perezi *(Seoane, 1885)* (manuscrito inédito).

Capítulo 6. *Analysis of the avoidance of nitrogen fertilizers in the water column by juvenile Iberian water frog,* Pelophylax perezi *(Seoane, 1885), in laboratory conditions* (manuscrito publicado bajo la forma: Egea-Serrano, A.; Tejedo, M. & Torralva, M. 2008. Analysis of the avoidance of nitrogen fertilizers in the water column by juvenile Iberian water frog, *Pelophylax perezi* (Seoane, 1885), in laboratory conditions. Bulletin of Environmental Contamination and Toxicology, 80: 178-183).

Bloque III.- Este bloque presenta los resultados obtenidos tras evaluar el impacto letal y subletal de cloruro amónico (aislado o combinado con nitrito sódico y nitrato sódico) en larvas de *P. perezi* en condiciones de mesocosmos. Del mismo modo, se incluyen los resultados obtenidos tras la exposición de larvas de *P. perezi* a localidades naturales que muestran un grado diferente de contaminación por compuestos nitrogenados. Incluye los siguientes capítulos:

Capítulo 7. *Effects of nitrogenous pollution on survival, morphology and growth of larvae of the Iberian waterfrog,* Pelophylax perezi *(Seoane, 1885): a hierarchical approach to natural conditions* (manuscrito inédito).

Capítulo 8. *Analysis of the effects of nitrogenous pollution on swimming performance of larvae of* Pelophylax perezi *(Seoane, 1885) through assays representative of natural conditions* (manuscrito inédito).

Bloque IV.- Este bloque incluye un único capítulo donde se analiza de modo global, mediante revisión bibliográfica y meta-análisis de los datos obtenidos, el impacto letal y subletal que la contaminación química ejerce sobre los anfibios.

Capítulo 9. *Are amphibians actually threatened by chemicals? A meta-analytic review* (manuscrito inédito).

Bloque V.- Este bloque presenta las principales conclusiones extraídas de los resultados presentados en los capítulos anteriores. Consta de un único capítulo y de su correspondiente traducción española.

Capítulo 10 (versión inglesa). *Conclusions* (manuscrito inédito).

Capítulo 10 (versión española). *Conclusiones* (manuscrito inédito).

BLOQUE I

INTRODUCCIÓN

CAPÍTULO 1

INTRODUCCIÓN Y OBJETIVOS

INTRODUCCIÓN

Aproximaciones conservadoras a la pérdida global de biodiversidad estiman tasas de extinción, incluyendo especies probablemente extintas, 102-1024 veces mayores a las esperadas en condiciones naturales (Baillie *et al.*, 2004), si bien otros estudios han estimado tasas 1000-11000 veces superiores (Pimm & Brooks, 1997). El incremento en varios órdenes de magnitud de las tasas de extinción respecto a condiciones naturales ha hecho que el objetivo de minimizar la pérdida global de biodiversidad, con los menores costes posibles para el desarrollo de la humanidad, sea uno de los principales problemas a afrontar en el siglo XXI (Wilson, 1994; Leakey & Lewin, 1997). Entre los vertebrados, los anfibios representan el grupo más amenazado (Baillie *et al.*, 2004), existiendo evidencias de la regresión de sus poblaciones en diferentes regiones del mundo (e.g. Corn & Flogeman, 1984; Pounds & Crump, 1994; Drost & Fellers, 1996; Laurance *et al.*¸ 1996; Lips, 1998; Galán, 1999; Bosch *et al.*, 2001; Martínez-Solano *et al.*, 2003a; Galán, 2008; Rovito *et al.*, 2009). No obstante, dado que las regiones Neotropical, Afrotropical e Indomalaya representan áreas con alta diversidad de anfibios para las que el número de estudios publicados es reducido (Brito, 2008), es probable que el número de poblaciones y especies actualmente reconocidas como amenazadas esté infravalorado.

A pesar del elevado grado de vulnerabilidad descrito para numerosas especies de anfibios, esta información debe ser interpretada con cautela, ya que las fluctuaciones demográficas naturales pueden ser la causa de las tendencias poblacionales observadas (Pechmann *et al.*, 1991; Tejedo, 2003). Así, estudios realizados durante series temporales cortas, o que adolecen de una baja frecuencia o intensidad de muestreo, pueden afectar severamente a la precisión de los resultados obtenidos (Alford & Richards, 1999). Adicionalmente, numerosas poblaciones de anfibios se comportan como metapoblaciones, lo que implica la existencia de flujo migratorio entre las diferentes poblaciones que las conforman (Alford & Richards, 1999; Joly *et al.*, 2001). De este modo, eventos de extinción y colonización podrían ser comunes, con lo que la constatación del declive de una población local podría formar parte de la dinámica metapoblacional natural, no correspondiendo por lo tanto a un auténtico riesgo de extinción. Todas estas consideraciones han llevado a la distinción entre tamaño poblacional y número de poblaciones, siendo necesario el reconocimiento de una tendencia regresiva en el número de poblaciones para poder identificar un auténtico riesgo de extinción (Green, 1997). Houlahan *et al.* (2000) resolvieron el debate planteado sobre la existencia de una auténtica disminución de las poblaciones de anfibios mediante el análisis cuantitativo de datos correspondientes a 936 poblaciones de todo el mundo y a series temporales de hasta 31 años. Este estudio pone en evidencia la existencia de una regresión generalizada de este grupo de vertebrados durante las últimas décadas con una notable variabilidad geográfica y temporal.

Los anfibios muestran una piel desnuda y permeable que los puede hacer altamente vulnerables a procesos de contaminación. Además, la explotación de hábitats tanto terrestres como acuáticos por la mayor parte de las especies para completar su ciclo vital las hace particularmente susceptibles a la alteración de cualquiera de estos medios.

Ambas consideraciones han sido utilizadas para sustentar la hipótesis de una especial vulnerabilidad de los anfibios respecto a otros grupos zoológicos frente a los cambios globales (Soccianti, 2001), los cuales se han descrito como factores responsables de su declive. No obstante, aunque agentes de ámbito global, como el cambio climático o la radiación ultravioleta (UV), han sido reconocidos como importantes amenazas para los anfibios, es muy destacable el efecto negativo producido por la desmesurada explotación de los recursos naturales por parte del hombre a una escala más limitada geográficamente. Dicha explotación afecta negativamente a las poblaciones de anfibios a través de diversas prácticas que, a pesar de sus efectos locales, están ampliamente distribuidas por todo el mundo. A continuación se describen de manera sucinta algunos de los principales factores, tanto globales como locales, reconocidos como amenazas para los anfibios (Alford & Richards, 1999; Blaustein & Kiesecker, 2002; Gardner, 2001; Collins & Storfer, 2003; Semlitsch, 2003; Beebee & Griffiths, 2005).

Cambios globales: Cambio climático y radiación UV

La emisión a la atmósfera de grandes cantidades de gases invernadero ha sido citada como causante del cambio climático que está sufriendo el planeta, caracterizado principalmente por el incremento de las temperaturas y el cambio del régimen de precipitaciones (IPCC, 2001). Dado que la temperatura y humedad ambiental son algunos de los factores desencadenantes de la reproducción de los anfibios (e.g. Carey & Alexander, 2003), el citado aumento de las temperaturas plantea la hipótesis de si los anfibios presentarán una tendencia a reproducirse antes. Así, aunque existen especies para las que esta tendencia no se ha detectado (Blaustein *et al.*, 2001a), otras sí que la presentan (Gibbs & Breisch, 2001). En este último caso, a pesar de que la modificación de la fenología reproductiva podría afectar a la supervivencia de embriones y larvas, sus

consecuencias a largo plazo son difíciles de predecir (Gibas & Breisch, 2001; Carey & Alexander, 2003). Adicionalmente, el aumento de las temperaturas, unido a irregularidades climáticas como prolongación de los períodos de sequía, puede incrementar las tasas de desecación de los cuerpos de agua, reduciendo su profundidad y exponiendo embriones y larvas de las diferentes especies a agentes ambientales estresantes, como la radiación ultravioleta (Kiesecker *et al.*, 2001; Blaustein & Kiesecker, 2002). En este sentido, se ha relacionado la vulnerabilidad de las especies de anfibios de Europa al cambio climático con la disponibilidad de agua, cuyos efectos se verían agravados por la limitada capacidad de dispersión de estas especies (Araújo *et al.*, 2006).

La emisión de compuestos químicos industriales (especialmente CFCs) a la atmósfera está implicada en los procesos responsables del adelgazamiento de la capa de ozono estratosférica (Solomon, 1999). Este adelgazamiento implica una insuficiente capacidad de filtración de la radiación ultravioleta, con el consiguiente incremento de su transmisión hasta la superficie de la tierra (Madronich *et al.*, 1998). Aunque se han identificado diferentes efectos adversos de la radiación ultravioleta sobre el tegumento, ADN, fotosíntesis o sistema inmune, la exposición a la radiación de longitudes de onda más corta (radiación UV-B [RUV-B], λ= 280-315 nm) es la que ocasiona consecuencias más severas (Madronich *et al.*, 1998). Así, se ha descrito un impacto negativo de la RUV-B sobre los organismos, tanto en ecosistemas terrestres como acuáticos (Caldwell *et al.*¸1998; Hädder *et al.*, 1998; Bancroft *et al.*¸ 2007). Por lo que respecta a los anfibios, la exposición a RUV-B produce efectos tanto letales como subletales, afectando al crecimiento, desarrollo, fisiología, anatomía y comportamiento de los individuos (Blaustein *et al.*, 2001b). Así, aunque existen variaciones inter- e intraespecíficas en relación a la sensibilidad a este tipo de radiación (Lizana & Pedraza,

1998; Broomhall *et al.*, 2000; Belden & Blaustein, 2002), el hecho de que de manera global la exposición a la RUV-B reduzca la supervivencia de los anfibios, independientemente del taxon o del estadio de desarrollo (Bancroft *et al.*, 2008), hace de éste uno de los principales factores implicados en el cambio global responsable del declive de los anfibios (Blaustein & Kiesecker, 2002; Blaustein *et al.*¸ 2003a, b).

Destrucción y alteración del hábitat

La destrucción del hábitat ha sido descrita como la principal causa de extinción de especies (e.g. Tilman *et al.*, 1994; Pimm & Raven, 2000). Entre los efectos producidos por la destrucción, y consecuente fragmentación, del hábitat se encuentran: 1) reducción inmediata del tamaño poblacional en relación a la cantidad de hábitat perdido (Andrén, 1994); 2) menores tasas migratorias como consecuencia del mayor aislamiento entre manchas de hábitats adecuados (Rukke, 2000; Dale, 2001; Virgos, 2001); 3) menor densidad poblacional debido a la disminución de la superficie de hábitat adecuado (Verboom *et al.*, 1991); 4) fluctuaciones en el número de nacimientos y muertes, así como de individuos emigrantes e inmigrantes (Fahrig, 2001, 2002), lo que disminuye la estabilidad demográfica de las poblaciones (Lacy & Lindenmayer, 1995; Lindenmayer & Lacy, 1995); 5) interrupción de los factores físicos y bióticos e incremento del efecto borde (Fagan *et al.*, 1999), lo que puede hacer que la distribución de las especies afectadas se restrinja a las zonas más naturales de las áreas afectadas por la fragmentación (i.e. núcleos de las manchas de hábitat inalterado) (Saunders *et al.*, 1991); y, 6) cambios en la biología, comportamiento e interacciones entre especies (Fischer & Lindenmayer, 2007). Todos estos factores conducen a que las poblaciones supervivientes en las manchas de hábitat inalterado estén amenazadas por procesos

demográficos, ambientales y genéticos estocásticos que pueden comprometer su viabilidad (Gilpin & Soulé, 1986; Lande, 1988).

Por lo que respecta a los anfibios, la tolerancia a la alteración del hábitat varía entre especies (Ficetola & De Bernardi, 2004). Por otra parte, la incidencia sobre las mismas de la alteración del hábitat varía geográficamente, estando afectadas principalmente las zonas del planeta con mayor riqueza específica (i.e. regiones subtropical y tropical) debido al crecimiento de la población humana (Gallant *et al.*, 2007). Sin embargo, su escasa capacidad de dispersión (Sinsch, 1990), así como las migraciones orientadas a la reproducción realizadas por muchas especies (Pope *et al.*, 2000), hacen que la destrucción y alteración del hábitat sea uno de los principales factores de amenaza que las poblaciones de de anfibios estén afrontando actualmente (Dodd & Smith, 2003).

La proliferación de explotaciones agrícolas, la silvicultura intensiva y el desarrollo urbanístico representan una de las principales causas responsables de la destrucción y alteración del hábitat. Esta transformación en los usos del suelo implica la eliminación directa de la vegetación natural, la homogeneización del terreno, y, a menudo, la desecación de humedales y sobreexplotación de acuíferos (Baraza, 2003; Martínez & Esteve, 2003). Paradójicamente, en áreas agrícolas dicha sobreexplotación tiene como consecuencia la creación de nuevos cuerpos de agua (i.e. balsas de riego), aunque su tipología y gestión los hace inadecuados para gran número de especies (Scoccianti, 2001). Como consecuencia, la disponibilidad de refugios y de cuerpos de agua es reducida (o corresponden a tipologías subóptimas). De este modo, aunque son varias las especies que pueden completar su ciclo vital en entornos agrícolas y urbanos (e.g. Beja & Alcazar, 2003; Vinces, 1993; Riley *et al.*, 2005; Rubbo & Kisecker, 2005; Husté *et al.*, 2006; Lane & Burgin, 2008), la escasa disponibilidad de refugio y de hábitats reproductores conduce a una reducida diversidad (pero ver Lane & Burgin, 2008).

Además, el uso de productos fitosanitarios tiene como consecuencia la contaminación de hábitats tanto terrestres como acuáticos, contribuyendo así a agudizar el impacto negativo de las explotaciones agrícolas sobre los anfibios (ver abajo).

El incremento de la superficie destinada a la agricultura o al desarrollo urbano implica la proliferación de las infraestructuras viarias que comunican estas áreas entre sí y que permiten la circulación dentro de ellas. Dicha proliferación contribuye en gran medida a la fragmentación del hábitat (Scoccianti, 2001). Por otra parte, diversas especies de anfibios pueden encontrarse en las carreteras como consecuencia de los desplazamientos realizados durante su actividad diaria, por la realización de migraciones reproductoras, la selección de las carreteras como rutas migratorias, su utilización como lugar de encuentro durante las épocas reproductoras (especialmente si existen cuerpos de agua cercanos) o por sus características microclimáticas (e.g. temperatura). Así, dada la escasa capacidad de movimiento de muchas especies, unido a su incapacidad para reconocer el peligro a tiempo y a que un mecanismo de defensa consiste en permanecer inmóvil, las carreteras representan la muerte por atropello de un gran número de ejemplares (Scoccianti, 2001; Dodd & Smith, 2003; Santos *et al.*, 2007; Sillero, 2008; Langen *et al.*, 2009). Así, pueden resultar severamente afectados factores como distribución de las especies, densidad y riqueza específica, abundancia de individuos, *sex - ratio* y flujo genético (Puky, 2005; Eidenbrod *et al.*, 2008).

Por otra parte, los incendios, voluntarios o accidentales, modifican las características físicas y químicas de las capas superficiales del suelo y eliminan la vegetación, lo que puede incrementar la tasa de erosión del suelo y la tasa de desecación y colmatación de los cuerpos de agua (Soccianti, 2001). Como consecuencia, los incendios tienen una notable capacidad para alterar el hábitat (Corn *et al.*, 2003). Este hecho, unido a los efectos directos (i.e. muerte de ejemplares) e indirectos (menor

disponibilidad de alimento, refugio o hábitat reproductor) sobre los anfibios, hace de los incendios un importante factor de amenaza para estos vertebrados (Scoccianti, 2001; Corn *et al*. 2003; Pilliod *et al.*, 2003).

Especies exóticas

Una especie exótica es aquélla que se encuentra fuera de su área de distribución natural, histórica o actual, como consecuencia de su introducción directa o indirecta por parte del hombre (IUCN, 2000). Adicionalmente, si actúa como agente de cambio del ecosistema natural o seminatural donde se ha establecido, o amenaza la diversidad biológica nativa, se define como especie invasora (IUCN, 2000).

La introducción de especies invasoras ha sido descrita como el primer o segundo impacto antrópico más importante que amenaza a los ecosistemas de agua dulce (Lodge *et al.*, 2000). La introducción, voluntaria o accidental, de una especie invasora implica: 1) alteraciones etológicas, del flujo genético y de la tasa de reproducción de los individuos pertenecientes a especies nativas; 2) presencia de fenómenos de competencia, depredación e hibridación; 3) modificación de la abundancia, distribución y estructura de las poblaciones, lo que puede incrementar la tasa de extinción; 4) alteración de la estructura y composición de las comunidades y reducción de la diversidad biológica; y, 5) cambios del medio físico (GEIB, 2006). La severidad de los efectos anteriores, así como la presencia permanente, al menos a escala ecológica, de las especies invasoras ha conducido a la masiva homogeneización biótica de la superficie de la Tierra resultante de la destrucción de las fronteras biogeográficas que han mantenido diferenciadas la flora y fauna características de las diferentes regiones del planeta (Kiesecker, 2003).

Por lo que a los anfibios respecta, la introducción de especies exóticas o invasoras ha conducido al declive de numerosas poblaciones como consecuencia del incremento de la mortalidad, presencia de alteraciones etológicas (e.g. disminución actividad, incremento del uso del refugio) o disminución del tamaño (ver revisión Kats & Ferrer, 2003). Las especies exóticas o invasoras capaces de depredar o competir sobre las especies de anfibios nativas incluye una gran variedad de taxa que comprende tanto invertebrados (e.g. *Procambarus clarkii* Girard, 1852) (Gamradt & Kats, 1996; Galán, 1997; Gamradt *et al.*, 1997; Cruz *et al.*, 2006), como vertebrados: peces (e.g. *Gambusia affinis* (Baird & Girard, 1853), *Lepomis gibbosus* (Linnaeus, 1758), *Micropterus dolomieui* Lacepède, 1802, *Oncorhynchus mykiss* (Walbaum, 1792), *Carassius auratus* (Linnaeus, 1758)) (Kiesecker & Blaustein, 1998; Galán, 1997; Goodsell & Kats, 1999; Adams, 2000; Martínez-Solano *et al.*, 2003b), reptiles (e.g. *Natrix maura* (Linnaeus, 1758)) (Moore *et al.*, 2004), anfibios (e.g. *Rhinella marina* (Linnaeus, 1758), *Lithobates catesbeianus* (Shaw, 1802), *Pelophylax perezi* (Seoane, 1885)) (Kiesecker & Blaustein, 1998; Lawler *et al.*, 1999; Moore *et al.*, 2004; Greenlees *et al.*, 2007). En este último caso, su presencia representa adicionalmente en algunos casos la posibilidad de que tengan lugar procesos de hibridación (Hotz *et al.*, 1994; Arano & Llorente, 1995; Crochet *et al.*, 1995; Pagano *et al.*, 2001), los cuales implican una importante amenaza para las especies parentales nativas como consecuencia de la contaminación genética que implican (Arano *et al.*, 1995). Además, los individuos híbridos pueden presentar altas tasas de fertilización y de viabilidad de larvas y embriones, e incluso mayor fecundidad y velocidad de crecimiento que las especies parentales (e.g. Hotz *et al.*, 1994) lo que puede conducir al desplazamiento y eventual desaparición de sus poblaciones.

Por otra parte, el declive de las poblaciones de anfibios como consecuencia de la introducción de especies exóticas no sólo es atribuible a especies animales, si no también a especies vegetales. Así, la modificación del hábitat como consecuencia de la plantación de eucaliptos puede tener importantes consecuencias sobre las poblaciones de anfibios nativas (Malkmus, 2004, pero ver Vences, 1993).

Enfermedades emergentes

Enfermedades descritas recientemente, que aparecen en poblaciones donde estaban ausentes o que incrementan su virulencia, incidencia o distribución geográfica se definen como enfermedades emergentes (Daszak *et al.*, 2003). La asociación entre la tasa de morbidez y mortalidad detectada en poblaciones silvestres de anuros de Centroamérica y Australia y la incidencia de cambios epidérmicos causados por hongos patógenos permitió identificar por primera vez la relación entre la regresión de las poblaciones de anfibios y la presencia de enfermedades emergentes (Berger *et al.*, 1998). A pesar de que dicha relación se detectó originalmente en áreas geográficas concretas, la incidencia actual de las enfermedades emergentes sobre los anfibios es global (AmphibiaWeb, 2009).

Aunque las enfermedades desarrollan un papel importante en la dinámica de poblaciones de animales y plantas (Anderson & May, 1986), es insólito el gran número de ecosistemas donde han sido detectadas las enfermedades emergentes en todo el mundo, así como el gran número de especies afectadas (Carey *et al.*, 2003). La virulencia de un agente patógeno depende tanto de las condiciones medioambientales como de la dinámica poblacional a escala local, metapoblacional o regional (Collins *et al.*, 2003). Así, actualmente se estudian dos hipótesis para explicar la incidencia de las enfermedades emergentes en los anfibios. En primer lugar, la dispersión de agentes

patógenos en nuevas áreas puede hacer que éstos afecten a nuevos huéspedes altamente susceptibles a la infección (*novel pathogen hypothesis*, NPH) (Alford, 2001). Una segunda hipótesis (*endemic pathogen hypothesis*) defiende que los agentes patógenos siempre han estado en el medio ambiente de los anfibios que son afectados, no habiendo sido detectados hasta tiempos recientes o teniendo lugar un incremento de su patogeneicidad como consecuencia de cambios medioambientales (ver revisión Rachowicz *et al.*, 2005). A pesar de la existencia de evidencias que defienden la NPH (e.g. Lips *et al.*, 2006), aún no se puede concluir que la descripción de enfermedades emergentes corresponde a la presencia de nuevos agentes patógenos, siendo necesaria la realización de más estudios genéticos para confirmar si la variación alélica de dichos agentes en áreas donde se consideran exóticos es menor a la detectada en las poblaciones fuente (ver revisión Rachowicz *et al*., 2005).

Entre los organismos responsables de las enfermedades emergentes en anfibios destacan hongos (*Batracochytrium* sp, *Saprolegnia* sp), trematodos parásitos (*Riberoia* sp) y virus (ranavirus) (ver revisión en Daszak *et al.*, 2003). La infección por *Batracochytrium* sp se ha reconocido como el ejemplo más notorio de la relación entre un agente patógeno y el declive de anfibios, siendo *Saprolegnia* sp otro organismo candidato como causa del declive de poblaciones de este grupo de vertebrados (ver revisión en Daszak *et al.*, 2003). *Batracochytrium* sp afecta a larvas y, especialmente, a ejemplares que están completando la metamorfosis debido a que este hongo degrada las células queratinizadas. Así, la hiperqueratosis e hiperplasticidad derivada de esta acción, junto a la producción de una toxina que difundiera a través de la piel hasta la sangre y/o otras alteraciones (e.g. alteración de la toma de iones y agua a través de la piel ventral), podrían explicar los severos efectos de la infección por *Batracochytrium* sp (Carey *et al.*, 2003). Adicionalmente, los efectos directos producidos por este hongo pueden

debilitar a los organismos afectados, lo que los puede hacer más susceptibles a infecciones por bacterias como *Aeromonas hydrophila* (Carey *et al.*, 2003), la cual ha sido asociada con mortalidades masivas de larvas (Márquez *et al.*, 1995) En el caso de *Saprolegnia* sp, la mortalidad tanto de embriones como de larvas y ejemplares metamórficos se incrementa como consecuencia de la infección por este agente patógeno (Carey *et al.*, 2003; Fernández-Benéitez *et al.*, 2008). La causa de la muerte ha sido atribuida a fallo orgánico debido a la necrosis aguda de tejidos hematopoyéticos y linfoides, hígado, riñones, músculo y tracto digestivo (Carey *et al.*, 2003). Por otra parte, aunque las infecciones por *Riberoia* sp y por ranavirus incrementan la tasa de malformaciones en ejemplares metamórficos y la mortalidad, respectivamente, en las poblaciones, hasta el momento no se ha descrito su asociación con el declive de anfibios (ver revisión en Daszak *et al.*, 2003). No obstante, ello no implica que no puedan tener afectos adversos sobre sus poblaciones, siendo necesario por ello tomar las medidas preventivas oportunas para limitar su dispersión.

Aunque la dispersión de las enfermedades emergentes puede ser debida a causas naturales (e.g. otros animales), el hombre es la causa más probable (Carey *et al.*, 2003). Actividades como el transporte de agua, barro o animales de zoológicos, la acuicultura, la utilización de anfibios como cebo, el comercio de anfibios, la liberación voluntaria o inadvertida de mascotas o de animales utilizados en la experimentación o como agentes de biocontrol pueden expandir los agentes patógenos responsables de las enfermedades (Carey *et al.*¸ 2003; Collins *et al.*, 2003; Daszak *et al.*¸ 2003; Picco & Collins, 2008).

Sobreexplotación y muerte intencionada

Los anfibios forman parte de la gastronomía de diversas culturas. A escala global, el número de especies utilizadas para el consumo humano (incluidas algunas amenazadas)

asciende a 212 en el caso del consumo de subsistencia o del mercado local (i.e. aquél que incluye el trueque por otros bienes, pero no la venta para obtener ganancias), siendo 66 y 20 las especies comercializadas a escalas subnacional/nacional (i.e. venta para obtener ganancias sin cruzar fronteras internacionales) e internacional (i.e. venta para obtener ganancias cruzando una o más fronteras internacionales), respectivamente (Carpenter *et al.*, 2007). Aunque algunas especies de salamandras han sido incluidas en la cultura culinaria de algunas regiones (Fitzgerald, 1989), las ancas de rana representan la forma mayoritaria de consumo de anfibios, siendo utilizadas particularmente especies de tamaño medio o grande (Jensen & Camp, 2003). En este último caso, el volumen de ejemplares capturados y exportados asciende a miles de toneladas (ver revisión Warkentin *et al.*, 2009). La selección de tallas, así como la eficacia y el momento de la colección de los ejemplares, puede afectar negativamente a la capacidad de recuperación de las poblaciones (Jennings *et al.*, 1999), lo que, unido a la gran cantidad de ejemplares capturados, conduciría al declive de las poblaciones locales, tal y como ocurrió a las poblaciones de *Pelophylax kl. esculentus* (Linnaeus, 1758) en Europa (Carpenter *et al.*, 2007).

Por otra parte, los anfibios han sido utilizados para llevar a cabo actividades educativas e investigadoras (Jensen & Camp, 2003). Ello ha llevado a la aparición de empresas comerciales especializadas proveedoras de estos vertebrados. Sin embargo, la mayor parte de ellas captura los individuos a comercializar en poblaciones silvestres, lo que, al menos a escala local, puede tener un efecto negativo a pesar de que se defina su captura como "sostenible". Aunque el aumento de las preocupaciones éticas (y consiguientes restricciones legales) en relación a la realización de disecciones ha disminuido su práctica en los programas académicos, los anfibios evidentemente se siguen empleando en investigación. Los objetivos particulares de cada estudio pueden

exigir que el material biológico utilizado proceda de poblaciones silvestres, lo que puede producir efectos negativos, especialmente en el caso de especies con áreas de distribución pequeñas o con poblaciones aisladas o pequeñas (Jensen & Camp, 2003). Sin embargo, el impacto de la colección científica en las poblaciones de anfibios es actualmente desconocido (Jensen & Camp, 2003).

La farmacopea tradicional de diversas culturas ha utilizado a los anfibios por sus propiedades medicinales o afrodisíacas (Jensen & Camp, 2003). Adicionalmente, la medicina occidental ha utilizado los anfibios desde fecha más reciente para la realización de pruebas de embarazo (Hansen, 1960) y para la búsqueda de nuevos medicamentos (Chivian & Bernstein, 2008a). Aunque el impacto del uso de los anfibios con fines medicinales no se conoce, la obtención de ejemplares de la naturaleza (como en el caso de la medicina china) y la investigación y consiguiente producción de productos ampliamente comercializados podría tener un gran impacto sobre las poblaciones silvestres (Jensen & Camp, 2003).

La brillante coloración de los anfibios, y en el caso de algunas especies su rareza, ha hecho que se haya incrementado el interés por este grupo de vertebrados entre los aficionados a la terriofilia, lo que ha fomentado a su vez su comercio. Por ejemplo, en Florida (Estados Unidos) durante el período 1990-1992 se han comercializado 1050 salamandras y 41500 anuros, llegando a alcanzarse tasas anuales de hasta 54000 hílidos en otras partes de los Estados Unidos (Louisiana) (Jensen & Camp, 2003). Esta importante presión sugiere que sus efectos negativos sobre las poblaciones silvestres de anfibios pueden ser importantes, al menos a escala local o regional.

Aunque su práctica no está excesivamente extendida, existen actividades adicionales a las anteriormente mencionadas que implican la captura de ejemplares. Así, el uso de diversas especies de anfibios como cebo para pesca (Meronek *et al.*, 1997;

Picco & Collins, 2008) o para la elaboración de souvenirs y, en el caso de especies con pieles gruesas, su utilización en la industria peletera (Soccianti, 2001; Jensen & Camp, 2003) puede producir un impacto local sobre la viabilidad de poblaciones silvestres, aunque insignificante en comparación con los usos anteriormente citados.

Por último, la persecución directa por parte del hombre como consecuencia de una aversión secular a los anfibios conduce a la muerte de ejemplares que, salvo ocasiones puntuales, tiene un carácter anecdótico ya que, las costumbres crípticas y nocturnas de la mayor parte de las especies las hace difícilmente detectables.

Acidificación y contaminación química

Gran número de actividades humanas tiene como consecuencia el vertido, voluntario o no, de una amplia gama de sustancias químicas tóxicas al medio (e.g. metales pesados, fertilizantes, pesticidas) que pueden tener efectos locales o ser transportadas a través de la atmósfera largas distancias (Blaustein & Kiesecker, 2002; Fernández & Grimalt, 2003). Como consecuencia, la contaminación se ha descrito como uno de los factores que amenazan más severamente a la biodiversidad actual (Chivian & Bernstein, 2008b).

Por lo que respecta a los anfibios, la contaminación por diversas sustancias ha sido relacionada con el declive de diversas poblaciones en diferentes territorios (Berger, 1989; Davidson *et al.*, 2001; Sparling *et al.*, 2001; Fellers *et al.*, 2004; Hamer *et al.*, 2004), a pesar de que los datos existentes podrían no ser suficientes para determinar su impacto sobre las poblaciones a largo plazo (Bishop, 1992; Hall & Henry, 1992). Ello ha llevado a su reconocimiento como una de las causas potenciales del declive de las poblaciones de anfibios (Alford & Richards, 1999; Blaustein & Kiesecker, 2002). No obstante, su papel en la crisis que las poblaciones de anfibios están sufriendo podría estar subestimado, dado que actualmente la mayor parte de los estudios

ecotoxicológicos se han realizado con especies comunes presentes en países localizados en las regiones paleártica y neártica, a pesar de que en los países de las regiones indomalaya, afrotropical y neotropical son los que presentan mayor número de especies en regresión (Schiesari *et al.*, 2007).

Los procesos biogeoquímicos condicionan que hábitats tanto terrestres como acuáticos puedan estar expuestos a pH ácidos de manera natural (Rowe & Freda, 2000). Sin embargo, vertidos mineros y la emisión de óxidos de nitrógeno y azufre a la atmósfera en áreas industriales y urbanas como consecuencia de la quema de combustibles fósiles incrementan la acidez del medio tanto por el vertido de residuos ácidos como por el depósito atmosférico (i.e. lluvia ácida) (Rowe & Freda, 2000; Scoccianti, 2001). A pesar de que algunas especies pueden explotar ambientes naturalmente ácidos para completar su ciclo biológico (e.g. Räsänen *et al.*¸ 2003), la expansión de entornos ácidos como consecuencia de actividades antrópicas ha sido reconocida como una causa potencial del declive de anfibios (Scoccianti, 2001; Blaustein *et al.*, 2003b). Estudios experimentales han demostrado que la exposición a pH bajo conduce al incremento de la mortalidad de las fases tanto acuáticas como terrestres del ciclo de vida de los anfibios (e.g. Räsänen *et al.*, 2003; D´Amen *et al.*, 2007). Adicionalmente, se ha asociado experimentalmente la acidez a efectos subletales entre los que se encuentran pérdida de sodio corporal, aceleración o retraso del desarrollo embrionario, disminución del tamaño y modificaciones etológicas (e.g. evitación de medios ácidos, disminución de la eficacia de protección de la freza) (Freda & Dunson, 1984; Bradford *et al.*, 1992; Freda & Taylor, 1992; Räsänen *et al.*, 2003; D´Amen *et al.*, 2007; Ortiz-Santaliestra *et al.*, 2007). Las severas consecuencias que estos efectos pueden tener sobre la viabilidad de los individuos expuestos a pH bajo y la capacidad de las fases terrestres del ciclo de vida de los anfibios para seleccionar

hábitats adecuados podrían contribuir a explicar la decadencia de algunas poblaciones de anfibios. Sin embargo, son varios los estudios que cuestionan esta vinculación (Rowe & Freda, 2000; Scoccianti, 2001), a pesar de que se ha relacionado el declive de una población de *Ambystoma tigrinum* (Green, 1825) en las Montañas Rocosas (Estados Unidos) con episodios de acidificación (Harte & Hoffman, 1989). Así, son necesarias nuevas evidencias para establecer el auténtico papel de la acidificación en el declive de los anfibios (Rowe & Freda, 2000).

Junto al impacto de la acidificación, los compuestos químicos cuyos efectos sobre los anfibios han sido más ampliamente estudiados hasta el momento corresponden a metales pesados, pesticidas y contaminantes orgánicos (Sparling *et al.*, 2000).

Los metales pesados representan el grupo químico cuyo efecto sobre los anfibios ha sido más ampliamente estudiado, probablemente como consecuencia de su impacto ecológico y del bajo coste de su análisis (Linder & Grillitsch, 2000). Los metales pesados engloban aquellos elementos metálicos con pesos atómicos superiores a 40 y una distribución electrónica similar en su capa externa (Linder & Grillitsch, 2000). Aunque su presencia puede ser consecuencia de la erosión de rocas y de erupciones volcánicas, las actividades agrícolas, mineras e industriales son las principales responsables de la presencia de estos metales en diferentes ecosistemas de todo el mundo (Linder & Grillitsch, 2000). Su biodisponibilidad está condicionada por numerosos factores tales como el contenido de carbono orgánico de los sedimentos, el tipo de arcilla que los constituye, su capacidad de intercambio catiónico, el potencial redox y el pH (Knezovitch *et al.*, 1987). Así, en ciertas condiciones los anfibios pueden estar expuestos a los metales pesados a través del consumo de alimento y el intercambio gaseoso cutáneo o pulmonar (Linder & Grillitsch, 2000; Unrine *et al.*, 2004). Como consecuencia de esta exposición, se han descrito incrementos en las tasas de mortalidad

y de malformación, tasas de crecimiento y desarrollo inferiores y menor aptitud de huida frente a depredadores (e.g. Unrine *et al.*, 2004; Fort *et al.*, 2006; Sparling *et al.*, 2006; García-Muñoz *et al.*, 2008; Marques *et al.*, 2008). Adicionalmente, el carácter liposoluble de los metales pesados puede conducir a su bioacumulación en los individuos expuestos (e.g. Tejedo & Reques, 2003; Hofer *et al.*¸ 2005), lo que puede convertir a las especies tolerantes a la contaminación en una amenaza para sus depredadores (Sparling & Lowe, 1996).

Los pesticidas incluyen un gran número de compuestos químicos diseñados para matar formas de vida específicas (e.g. insectos, hongos, plantas) (Scoccianti, 2001). Su utilización agrícola y doméstica representa importantes fuentes de estas sustancias (Scoccianti, 2001; Boone & Bridges, 2003). Hasta la formalización de las restricciones para el uso de pesticidas organoclorados (e.g. DDT) en los años 1970s y 1980s, esta familia de pesticidas se usó ampliamente (Boone & Bridges, 2003) Sus nocivos efectos, unido a su alta persistencia en el medio y su acumulación en las reservas lipídicas de los organismos, hizo que los pesticidas organoclorados fueran sustituidos por una gran diversidad de compuestos de nueva generación con menor persistencia e impacto en el medio. Sin embargo, ello no implica que estos nuevos pesticidas sean inocuos para la vida silvestre, ya que su toxicidad sobre los anfibios ha sido reconocida (ver revisión Cowman & Mazanti, 2000). En cualquier caso, la exposición a las diferentes familias de pesticidas produce una gran diversidad de efectos sobre los anfibios, entre los que se encuentra el incremento de la mortalidad, reducción de las tasas de crecimiento y desarrollo, mayor incidencia de malformaciones, alteraciones fisiológicas y disminución de los niveles de actividad, capacidad locomotora y tasa reproductora (e.g. Bridges, 1997; Bridges & Semlitsch, 2000; Allran & Karasov, 2001; Widder & Bidwell, 2008). Como consecuencia del impacto negativo que estos efectos tiene sobre la viabilidad de

los individuos afectados y, eventualmente, las poblaciones, la contaminación por pesticidas ha sido descrita como responsable de mortalidades masivas de anfibios (Davidson *et al.*, 2001; Sparling *et al.*, 2001; Fellers *et al.*, 2004). Adicionalmente, como en el caso de los metales pesados, su bioacumulación (e.g. Fellers *et al.*¸2004; Fagotti *et al.*, 2005; Hofer *et al.*¸ 2005) puede afectar negativamente a los depredadores de las especies tolerantes.

Otro grupo de contaminantes ampliamente considerados en estudios ecotoxicológicos son los contaminantes orgánicos. Dichos contaminantes engloban sustancias como bifenilos policlorados (PCBs), dioxinas, furanos o hidrocarburos aromáticos policíclicos (PAHs) (Sparling, 2000). Aunque muchos de estos compuestos actualmente no se utilizan en países industrializados, su uso industrial en países menos desarrollados y la emisión de PAHs como consecuencia de procesos industriales y de las emisiones de vehículos de motor convierten su presencia en ubicua (Sparling, 2000). Los contaminantes orgánicos se caracterizan por una gran persistencia en el medio y en los tejidos (con lo que se pueden incorporar fácilmente a la cadena trófica), así como por poder ser transportados a través de la atmósfera largas distancias (Sparling, 2000). Como en los casos anteriores, la exposición de los anfibios a esta categoría de contaminantes produce una gran variedad de efectos que comprenden tanto el incremento de las tasas de mortalidad de los individuos expuestos como disrupción endocrina, alteraciones fisiológicas, reducción de las tasas de desarrollo y malformaciones (Sparling, 2000; Marquis *et al.*, 2006).

Por otra parte, a pesar de la dominancia en los estudios ecotoxicológicos de los grupos de contaminantes anteriormente mencionados, otros compuestos pueden afectar negativamente a los individuos expuestos. Así, se ha identificado, mediante estudios experimentales, el impacto negativo que sustancias como sales descongelantes o

residuos de medicamentos presentes en los vertidos urbanos pueden tener sobre la supervivencia, actividad, crecimiento y desarrollo de embriones y larvas de anfibios (Fraker & Smith, 2004, 2005; Dougherty & Smith, 2006; Sanzo & Hecnar, 2006; Petterson & Berg, 2007; Karraker *et al.*, 2008). Sin embargo, entre los contaminantes cuyo efecto sobre los anfibios está despertando el interés de los científicos destacan los fertilizantes y otros compuestos nitrogenados, siendo creciente el número de publicaciones destinadas a establecer su efecto (ver revisión Marco & Ortiz-Santaliestra, en prensa).

Las prácticas agrícolas han sido reconocidas como la principal fuente de agentes contaminantes, especialmente de pesticidas y fertilizantes (Scoccianti, 2001). Sin embargo, otros factores (vertidos de aguas residuales procedentes de ciudades y explotaciones ganaderas, quema de combustibles fósiles e incendios) representan importantes fuentes adicionales de compuestos nitrogenados (Vitousek *et al.*, 1997; Ritter & Bergstrim, 2001; Pilliod *et al.*, 2003). A pesar de la escala local a la que todas las actividades anteriormente citadas ejercen sus efectos, la gran extensión del planeta donde son llevadas a cabo hace que el impacto de compuestos nitrogenados esté ampliamente extendido (Carpenter *et al.*, 1998), y que se espere su incremento en el futuro (Tilman *et al.*, 2001; Galloway *et al.*, 2003). Por lo que respecta a los anfibios, se han realizado estudios experimentales que han asociado efectos tanto letales como subletales a la exposición a diferentes compuestos nitrogenados. Junto al incremento de la mortalidad de los individuos expuestos, se han descrito alteraciones del crecimiento y desarrollo, alteraciones de las tasas ventilatorias, incremento de la incidencia de malformaciones y cambios etológicos (e.g. evitación de medios contaminados, reducción de la tasa de actividad, disminución de la tasa de consumo de alimento) tras la exposición a nitrógeno reactivo (ver revisión Marco & Ortiz-Santaliestra, en prensa).

Así, el impacto de estos efectos deletéreos puede contribuir a explicar el declive de las poblaciones de anfibios asociado a los compuestos nitrogenados (Berger, 1989; Hamer *et al.*, 2004).

Finalmente, hay que destacar que los efectos descritos para las diferentes categorías de contaminantes citadas corresponden a efectos directos. Sin embargo, estas sustancias pueden producir efectos indirectos sobre los anfibios, los cuales redundan en su impacto. Por ejemplo, la adición de nitrógeno inorgánico a ecosistemas dulceacuícolas conduce a su eutrofización. Como consecuencia, las características fisicoquímicas de la columna de agua cambian (e.g. reducción de la disponibilidad de luz y de oxígeno disuelto, formación de compuestos químicos tóxicos) y se incrementa la producción y biomasa del fitoplancton, lo que disminuye la riqueza específica botánica y zoológica del ecosistema (ver revisión Camargo & Alonso, 2006). Además, la estimulación del crecimiento algal por la incorporación de formas de nitrógeno reactivo al sistema puede conducir a la formación de taxa productores de toxinas intra- y extracelulares (ver revisión Camargo & Alonso, 2006), algunas de las cuales pueden afectar negativamente al crecimiento y desarrollo de algunas especies de anfibios (e.g. Oberemm *et al.*, 1999). Por otra parte, la presencia de contaminantes en el ecosistema puede modificar las relaciones tróficas mediante la alteración de la disponibilidad de alimento y la vulnerabilidad frente a depredadores naturales resistentes a la contaminación. Así, las evidencias disponibles en relación a los anfibios indican que éstos están afectados negativamente por este efecto indirecto de la contaminación (ver revisión Rouse *et al.*, 1999), aunque en algunos casos se han observado efectos beneficiosos sobre el crecimiento (e.g. Boone *et al.*, 2007).

Combinación de factores

Los anfibios están expuestos en sus ambientes naturales a la combinación de diferentes factores de amenaza. Así, estos factores estresantes pueden ejercer sus efectos a través de complejas interacciones (Blaustein & Kiesecker, 2002), las cuales pueden tener consecuencias aditivas o sinérgicas (Berenbaum, 1989). Así, factores de amenaza globales como el calentamiento de la superficie terrestre pueden incrementar la tasa de desecación de los cuerpos de agua donde se desarrollan embriones y larvas de muchas especies, lo que los expone a la RUV-B aumentando su vulnerabilidad a las infecciones (Kiesecker *et al.*, 2001; Blaustein & Kiesecker, 2002) y a contaminantes (Hatch & Blaustein, 2000; Baud & Beck, 2005; Macías *et al.*, 2007). Adicionalmente, la acción conjunta de factores que actúan a escala local puede exacerbar sus efectos sobre los anfibios. De este modo, la combinación de contaminantes con factores tanto abióticos (e.g. pH, otros contaminantes, luz) como bióticos (e.g. peces, hongos) puede incrementar la vulnerabilidad de los anfibios respecto a los efectos de estos factores de manera aislada (e.g. Fernández & L´Haridon, 1992; Romansic *et al.*, 2006; Boone *et al.*, 2007; Ortiz-Santaliestra, 2008).

Los aspectos anteriormente mencionados ponen de manifiesto que la vinculación del declive de los anfibios a factores aislados sea excesivamente simplista (Gardner, 2001; Blaustein & Kiesecker, 2002; Beebee & Griffiths, 2005). Así, el estudio del efecto de las interacciones entre factores bióticos (e.g. agentes patógenos, especies introducidas) y abióticos que actúan a escala global (e.g. cambio climáticos, RUV-B), regional (e.g. contaminación, acidificación) y local (e.g. alteración del hábitat) es esencial para establecer con mayor precisión las causas de la decadencia de este grupo de vertebrados (Gardner, 2001; Blaustein & Kiesecker, 2002; Storfer, 2003).

Las poblaciones de anfibios españolas: descripción y factores de amenaza

Como consecuencia de la realización de inventarios nacionales y regionales de especies de anfibios (e.g. Pleguezuelos *et al.*, 2002; Díaz-Paniagua *et al.*, 2005, García & Lizana, 2007; Fernández, 2008), estudios genéticos y morfológicos (e.g. Sánchez-Herráiz *et al.*, 2000) y cambios nomenclaturales y taxonómicos (Montori *et al.*, 2005; Carretero *et al.*, 2009), actualmente en España se ha reconocido la existencia de 35 especies de anfibios (11 especies de urodelos; 24 especies de anuros) (Carretero *et al.*, 2009).

La alta riqueza específica presente en España puede ser debida tanto al papel que la Península Ibérica jugó como refugio durante los eventos glaciales cuaternarios, como a la existencia de paleoendemismos más antiguos (Vargas & Real, 1997). Casi un tercio de las especies presentes en España son endemismos peninsulares (Vargas & Real, 1997). Se ha descrito la existencia en España de cuatro corotipos, uno de los cuales comprende especies ampliamente distribuidas por toda la superficie peninsular, mientras que los tres restantes incluyen especies de distribución septentrional. Esta segregación señala la existencia de una frontera norte-sur más permeable en dirección sur-norte debido a que la mayor irregularidad pluviométrica presente en la zona sur es un factor limitante para la supervivencia de las especies septentrionales. Adicionalmente, la distribución de urodelos y anuros a escala de grandes cuencas ibéricas está condicionada por parámetros ambientales distintos. Así, la incidencia de inundaciones determina la distribución de urodelos y la disponibilidad de energía ambiental la de anuros (Vargas & Real, 1997).

En España, la mayor parte de especies de anfibios son eurihipsas, estando presentes desde el nivel del mar hasta 2000 m.s.n.m., aproximadamente (Pleguezuelos & Villafranca, 1997). Sin embargo, hay cinco especies que se distribuyen hasta cotas inferiores a los 1200 m.s.n.m. (*Discoglossus pictus* (Otth, 1837), *Chioglossa lusitanica*

Bocage, 1864, *Alytes cisternasii* Bosca, 1879, *Hyla meridionalis* Boettger, 1874 y *Rana dalmatina* Bonaparte, 1840) y tres montanas que están ausentes a nivel del mar (*Alytes dickhilleni* Arntzen & García-París, 1995, *Calotriton asper* (Dugès, 1852) y *Rana pyrenaica* Serra-Cobo, 1993) (Pleguezuelos & Villafranca, 1997). La riqueza específica está correlacionada negativamente con la altitud, aumentando ligeramente el número de especies desde el nivel del mar hasta los 600 m.s.n.m. y disminuyendo ligeramente hasta los 2200 m.s.n.m. y de manera más pronunciada a partir de cotas más altas (Pleguezuelos & Villafranca, 1997).

El gran número de especies de anfibios presentes en España hace que la diversidad en relación a sus características biológicas y ecológicas sea elevada (ver revisión García-París *et al.*, 2004). Así, existen especies que, una vez concluida la metamorfosis, son terrestres, mientras que otras muestran hábitos estrictamente acuáticos (ver revisión García-París *et al.*, 2004). En cualquier caso, todas ellas necesitan de habitats acuáticos para completar su desarrollo larvario (ver revisión García-París *et al.*, 2004). La precipitación ha sido descrita como uno de los factores desencadenantes de la reproducción de buen número de especies (Díaz-Paniagua, 1986). Así, numerosas especies se reproducen fundamentalmente en primavera, aunque otras lo hacen más tardíamente, a finales de primavera o comienzos del verano (ver revisión García-París *et al.*, 2004). La duración del desarrollo larvario es muy variable en función de la especie y población (ver revisión García-París *et al.*, 2004), estando asimismo condicionado por características del hábitat como el hidroperíodo (e.g. Richter-Boix *et al.*, 2006). El período de actividad anual varía en función de la especie. La mayor parte de las especies son nocturnas, aunque algunas de ellas, como *P. perezi*, pueden presentar actividad tanto diurna como nocturna. La dieta de los individuos adultos está constituida principalmente por invertebrados tanto en el caso de anuros como de urodelos, aunque

en algunos casos se ha confirmado el consumo tanto de vertebrados como de carroña para especies como *P. perezi* (ver revisión Egea-Serrano, 2009). Las larvas de urodelos son depredadoras, mientras que la dieta de las larvas de anuros está constituida fundamentalmente por algas. En España, la longevidad de las especies presentes muestra una gran variabilidad, estando comprendida entre 5 y 30 años, aproximadamente (ver revisión García-París *et al.*, 2004). Junto a estudios relacionados con la reproducción, hábitat o actividad de las especies presentes en España, se han publicado estudios que revelan la capacidad de orientación de los individuos adultos de *Lissotriton helveticus* (Razoumowsky, 1789) según señales acústicas de especies simpátricas o según el campo magnético terrestre (al igual que en el caso de *Mesotriton alpestris* (Laurenti, 1768)). Asimismo, existen evidencias sobre la capacidad que tienen larvas de *P. perezi* para orientarse según el campo magnético terrestre y para reconocer la presencia de depredadores, así como sobre la influencia que las características del microhábitat, la talla y la existencia de grupos ejerce sobre el comportamiento de huida de los adultos de esta última especie de anuro (Diego-Rasilla *et al.*, 2005, 2008; Martín *et al.*, 2005, 2006; Gonzalo *et al.*, 2006, 2007, 2009; Diego-Rasilla & Luengo, 2007; Diego-Rasilla & Phillips, 2007).

La mayor parte de las especies de anfibios presentes en España se encuentran expuestas a un grado de vulnerabilidad a la extinción elevado (Pleguezuelos *et al.*, 2002a), habiéndose constatado el declive de diversas poblaciones (Galán, 1999, 2008; Bosch *et al.*, 2001; Martínez-Solano *et al.*, 2003a). Como consecuencia, se han revisado y descrito los principales factores que amenazan a la herpetofauna en general y a los anfibios en particular en España (Bosch & Ayllón, 1997; Pleguezuelos *et al.*, 2002b). Dichos factores incluyen aspectos como la alteración y destrucción del hábitat, contaminación, incendios, desarrollo de infraestructuras viarias, introducción de

especies exóticas y presencia de enfermedades emergentes, la mayor parte de los cuales han sido relacionados con el declive de las poblaciones de anfibios a escala global (e.g. Beebee & Griffiths, 2005).

Previamente se han señalado los efectos adversos que la destrucción y alteración del hábitat ejercen sobre los anfibios. En España, a pesar de haberse reconocido que la modificación del hábitat y los incendios son unas de las principales amenazas a las que están expuestos los anfibios (Bosh & Ayllón, 1997, Pleguezuelos *et al.*, 2002b), el número de publicaciones que analizan su efecto es muy escaso. Sin embargo, Galán (1997) describió que, a pesar de la restauración del hábitat en áreas cubiertas por materiales desechados en actividades mineras, especies con requerimiento ecológicos específicos como *C. lusitanica* desaparecieron. Además, Montori *et al.* (2007) mostraron que el número de especies presentes y, especialmente, que se reproducen en un cuerpo de agua dado se ve afectado negativamente por los incendios. Del mismo modo, la muerte de numerosos ejemplares de diversas especies de anuros y urodelos atropellados (Carretero & Rosell, 2000; Santos *et al.*, 2007; Sillero, 2008) pone de manifiesto el impacto negativo del incremento de la densidad del tráfico.

Son diversos los estudios que evidencian los efectos adversos que la introducción de especies exóticas (e.g. *P. clarkii, G. holbrooki, C. auratus*) ha tenido sobre diversas poblaciones españolas de anfibios (Galán, 1997b; Cruz *et al.*, 2006; Bermejo-García, 2007). Adicionalmente, la capacidad de hibridación entre especies nativas y exóticas (Hotz *et al.*, 1994; Arano & llorente, 1995; Crochet *et al.*, 1995) plantea la posibilidad de contaminación genética (Arano *et al.*, 1995), pudiendo ser los híbridos mejores competidores que las especies parentales (Hotz *et al.*, 1994), con lo que llegarían a desplazarlas.

La presencia de hongos patógenos y bacterias ha sido asociada con la regresión de poblaciones de *Bufo calamita* (Laurenti, 1768) y *Alytes obstetricans* (Laurenti, 1768) en las sierras de Guadarrama y Gredos (Márquez *et al.*, 1995; Bosch *et al.*, 2001; Martínez-Solano *et al.*, 2003a; Fernández-Benéitez *et al.*, 2008;). La relación entre mortalidades masivas de anfibios y hongos pone de manifiesto que estos organismos son importantes factores de amenaza, los cuales pueden estar comprometiendo gravemente a las poblaciones de diversas especies.

La contaminación de los hábitats naturales es uno de los principales factores de amenaza a los que se están enfrentando los anfibios españoles (Bosch & Ayllón, 1997; Pleguezuelos *et al.*, 2002b). Las actividades urbanas, agrícolas, industriales y mineras, actividades son las principales responsables de la presencia de metales pesados, pesticidas y fertilizantes en habitats naturales, así como de la acidificación del mismo (Linder & Grillitsch, 2000; Rowe & Freda, 2000, Scoccianti, 2001). Se han realizado estudios con poblaciones españolas de diversas especies que han puesto de manifiesto el impacto negativo que la exposición a estos compuestos, así como a entornos ácidos, produce sobre los individuos afectados al modificar su supervivencia, crecimiento, desarrollo, comportamiento e incluso al ser bioacumulados (e.g. Montori *et al.*, 1982; Álvarez *et al.*, 1995; Tejedo & Reques, 2003; Ortiz *et al*., 2004*;* Ortiz-Santaliestra *et al.*, 2007; García-Muñoz *et al.*, 2008). Sin embargo, el impacto de los diferentes contaminantes no siempre es idéntico, ya que se han detectado tanto diferencias ontogénicas como inter- e intraespecíficas para las especies de anfibios presentes en España (Ortiz-Santaliestra *et al.*, 2006; Shinn *et al.*¸ 2008).

Por otra parte, aunque los factores de amenaza descritos sean los que representan una amenaza más relevante para los anfibios (e.g. Beebee & Griffiths, 2005), existen otros cuyo efecto adverso puede ser de relevancia. Así, se ha descrito que agentes que

ejercen su impacto a escala mundial, como la radiación UV-B, incrementan significativamente la mortalidad en el caso de las poblaciones de *Bufo bufo* (Linnaeus, 1758) y *P. perezi* presentes en España (Lizana & Pedraza, 1998; Macías *et al.*, 2007).

El estudio del efecto que la interacción entre agentes estresantes produce sobre los anfibios reviste una gran relevancia, dado que el impacto de dicha interacción puede ser consecuencia de las complejas interacciones entre los diversos factores de amenaza que actúan conjuntamente (Blaustein & Kiesecker, 2002). Así, la combinación de compuestos nitrogenados y otros agentes estresantes (e.g. radiación UV-B, salinidad, hipoxia, alta temperatura) produce un efecto más severo que el impacto de estos factores por separado en el caso de especies como *Rana temporaria* Linnaeus, 1758, *B. bufo* o *P. perezi* (Macías *et al.*, 2007; Ortiz-Santaliestra, 2008). Sin embargo, la información existente sobre el impacto que la combinación de factores estresantes produce sobre las especies de anfibios presentes en España sigue siendo muy escasa.

Las poblaciones de anfibios en la Región de Murcia: descripción y factores de amenaza

En la Región de Murcia se ha descrito la presencia de ocho especies de anuros y una de urodelo (Egea-Serrano *et al.*, 2005a, b; Escoriza, 2005; Torralva *et al.*, 2005), si bien las especies *H. meridionalis* y *Pleurodeles waltl* Michahelles, 1830 han sido citadas en publicaciones más antiguas (Hernández-Gil *et al.*, 1993).

La Región de Murcia representa el límite de distribución oriental de las especies *A. dickhilleni* y *Salamandra salamandra* (Linnaeus, 1758), y occidental en el caso de *Alytes obstetricans*. Entre todas las especies presentes, *B. bufo*, *B. calamita*, *Pelodytes punctatus* (Daudin, 1802) y *P. perezi* son las que muestran una distribución regional más amplia (Egea-Serrano *et al.*, 2005a, b; Torralva *et al.*, 2005). Sin embargo, excepto

P. perezi, cuya área de distribución ocupa prácticamente toda la superficie regional, la distribución de estas especies está fragmentada (Egea-Serrano *et al.*, 2005a, b; Torralva *et al.*, 2005). Como consecuencia, se han identificado 103 cuadrículas U.T.M. 5 x 5 km distribuidas por toda la superficie regional (Fig. 1) cuya conservación debería ser prioritaria debido a su elevada riqueza específica y a la singularidad biológica de las especies presentes (Egea-Serrano *et al.*, 2006c).

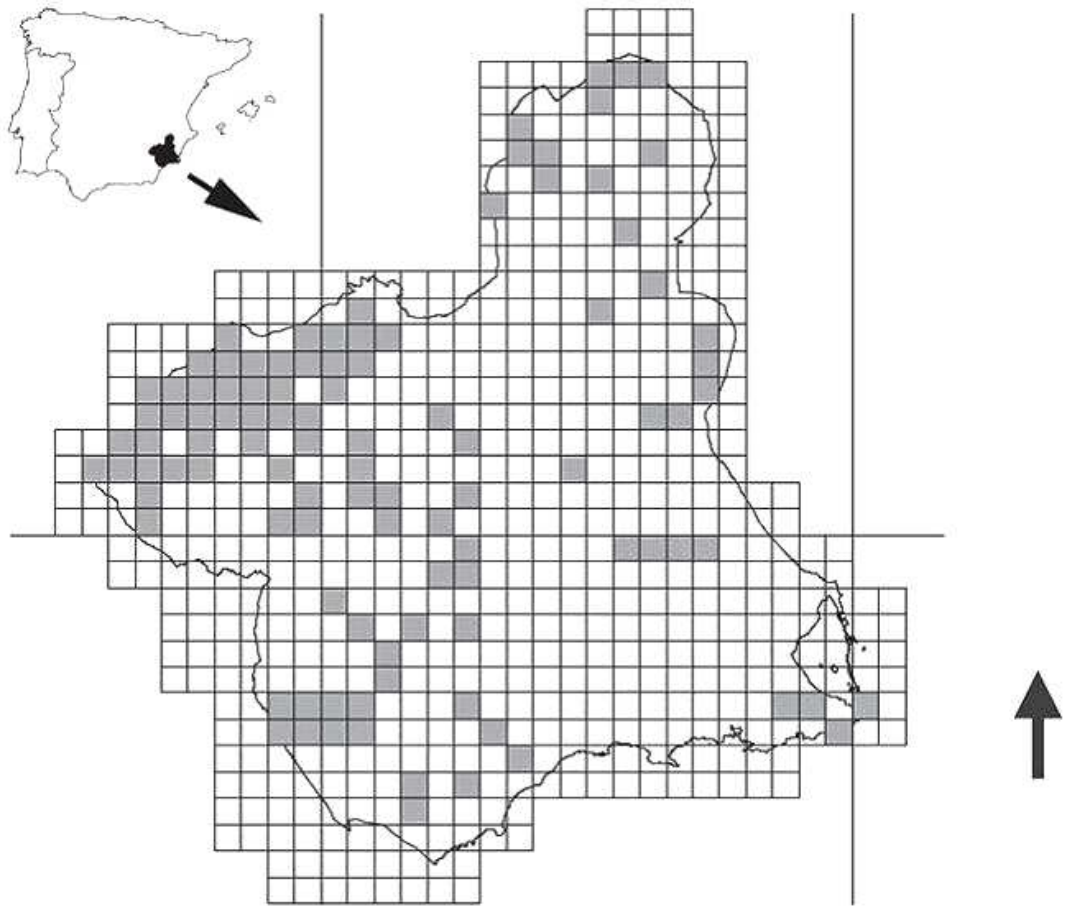

Fig. 1. Distribución de las áreas prioritarias de conservación para los anfibios en la Región de Murcia (modificado de Egea-Serrano *et al.*, 2006c).

El análisis de la distribución altitudinal a nivel regional de las especies de anfibios permitió identificar cuatro grupos de especies. Estos grupos diferencian especies estenohipsas de altitud media (*A. obstetricans*) y alta (*S. salamandra* y *A. dickhilleni*), especies se distribuyen a altitudes medias-altas (*B. bufo* y *P. punctatus*) y especies eurihipsas o que están presentes fundamentalmente a altitud media (*Pelobates cultripes* (Cuvier, 1829), *B. calamita* y *P. perezi*) (Egea-Serrano *et al.*, 2005c).

Los estudios relacionados con la biología y ecología de las poblaciones de anfibios presentes en la Región de Murcia son muy escasos. La información existente indica que cuerpos de agua correspondientes a las más variadas tipologías son utilizados como hábitats reproductores, aunque son las balsas de riego los ambientes utilizados con mayor frecuencia, debido probablemente a que en muchas áreas de la Región de Murcia representan los únicos cuerpos de agua disponibles (Egea-Serrano *et al.*, 2005a; Torralva *et al.*, 2005). Sin embargo, ambientes naturales, como arroyos, o tipologías utilizadas en las prácticas agropecuarias tradicionales (e.g. bebederos, albercas) son seleccionados positivamente por algunas de las especies estudiadas (Egea-Serrano *et al.*, 2005d; Egea-Serrano *et al.*, 2006a, b). En el caso de *S. salamandra* y *A. dickhilleni* se ha confirmado la selección positiva de localidades ubicadas en topografías montañosas o a altitudes superiores a 1250 m.s.n.m. (Egea-Serrano *et al.*, 2006a, b). Bajas concentraciones de sulfato en la columna de agua y la presencia de una cobertura de vegetación de ribera media o alta son seleccionadas positivamente por *S. salamandra* y *P. perezi*, respectivamente, a escala de microhábitat (Egea-Serrano *et al.*, 2005d; Egea-Serrano *et al.*, 2006a).

El período reproductor de *S. salamandra, A. dickhilleni* y *P. perezi* en la Región de Murcia es prolongado (*S. salamandra*: octubre-marzo; *A. dickhilleni*: febrero-julio, octubre; *P. perezi*: abril-julio), mientras que la reproducción de *B. bufo, B. calamita* y *P.*

punctatus se ha confirmado durante períodos de tiempo menos extensos (marzo-abril) (Egea-Serrano *et al.*, 2005e). La densidad larvaria relativa en el caso de *S. salamandra*, *A. dickhilleni* y *P. perezi* es superior en cuerpos de agua naturales o seminaturales (Egea-Serrano *et al.*, 2005e).

Las especies de anfibios más vulnerables a la extinción en la Región de Murcia son *A. dickhilleni*, *A. obstetricans* y *S. salamandra* (Egea-Serrano *et al.*, 2006c). Sin embargo, la aplicación de los criterios UICN a nivel regional (UICN, 2001, 2003) evidencia que las mayor parte de las especies, excepto *P. perezi*, *H. meridionalis* y *P. waltl*, se consideran vulnerables (Egea-Serrano *et al.*, 2007). Existen evidencias sobre el impacto que la exposición a compuestos nitrogenados produce sobre larvas de *P. perezi* (Egea-Serrano *et al.*, 2008, 2009a, b). Sin embargo, no se ha estudiado de manera pormenorizada el impacto que otros factores de amenaza puede tener sobre los anfibios a escala regional. No obstante, el análisis descriptivo de los factores de amenaza a los que están expuestos los cuerpos de agua presentes en la Región de Murcia evidencia que la modificación del medio acuático y la presencia de vertidos son las principales amenazas a las que están expuestos los anfibios durante su reproducción y desarrollo embrionario y larvario (Egea-Serrano *et al.*, 2005a; Torralva *et al.*, 2005).

JUSTIFICACIÓN DEL ESTUDIO Y OBJETIVOS

El número de estudios destinados a establecer el efecto de la contaminación sobre los anfibios es aún escaso, especialmente en relación a otros grupos faunísticos (Sparling *et al.*, 2000). No obstante, la información disponible hasta el momento ha permitido identificar diferentes tipos de sustancias, incluidos compuestos nitrogenados, como factores de amenaza para los anfibios (Sparling *et al.*, 2000; revisión Marco & Ortiz-

Santaliestra, en prensa). Sin embargo, el impacto de estas sustancias determinado a partir de dicha información podría estar desvirtuado ya que la mayor parte de los estudios ecotoxicológicos sobre anfibios consideran la acción de una sustancia tóxica de manera aislada (Storfer, 2003), lo cual no corresponde a un escenario natural (Blaustein & Kiesecker, 2002). Adicionalmente, la mayor parte de los estudios se han realizado en condiciones de laboratorio (Boone & James, 2005). Aunque los resultados obtenidos en estas condiciones representan un punto de partida importante para el establecimiento de los efectos de una sustancia, es necesario determinar si dichos efectos ocurren en ecosistemas naturales (Boone & Bridges, 2003; Boone & James, 2005). Estos aspectos, junto a las evidencias disponibles sobre la existencia de variabilidad inter e intraespecífica e incluso intrapoblacional en relación a la tolerancia a contaminantes como pesticidas (Bridges & Semlitsch, 2000; Widder & Bidwell, 2008) o compuestos nitrogenados (Marco *et al.*¸ 1999; Johansson *et al.*, 2001; de Wijer *et al.*, 2003), ponen de manifiesto la necesidad de realizar nuevas investigaciones que completen la base de datos mundial ya existente y que permitan establecer con precisión el auténtico impacto de la contaminación sobre los anfibios a escala mundial (Smith *et al.*, 2005). En este contexto, la presente Tesis Doctoral amplia la información existente sobre los efectos de los compuestos nitrogenados en los anfibios a través de una aproximación gradual a las condiciones naturales y profundiza en el impacto que los contaminantes químicos tienen sobre sus poblaciones.

Como fuente de nitrógeno reactivo fueron seleccionados los compuestos cloruro amónico (NH_4Cl), nitrito sódico ($NaNO_2$) y nitrato sódico ($NaNO_3$) debido a que: 1) tanto el cloruro amónico como, especialmente, el nitrato sódico son compuestos utilizados como fertilizantes agrícolas, lo que hace que sean importantes fuentes de nitrógeno reactivo, ya que la adición de fertilizantes a los campos agrícolas es una de las

principales fuentes de incorporación de nitrógeno al medio; 2) son fuente de distintos iones nitrogenados implicados en el ciclo global del nitrógeno y que difieren en sus efectos tóxicos; y, 3) las transformaciones químicas de estos compuestos liberan diferentes especies de nitrógeno reactivo por separado, pero no de manera conjunta, lo que los hace adecuados para estudiar el efecto de la combinación de diferentes concentraciones de las diferentes formas de nitrógeno. Se propuso la exposición a los compuestos anteriores en laboratorio y mesocosmos. Adicionalmente, los experimentos realizados fueron completados con la exposición a localidades naturales, en la que se analizó el efecto de los compuestos nitrogenados en condiciones completamente silvestres, y con una revisión bibliográfica en la que se evaluó el efecto global de los compuestos nitrogenados y otros contaminantes químicos en los anfibios.

Así, los objetivos concretos de la presente Tesis Doctoral fueron:

- Analizar el efecto del cloruro amónico, nitrito sódico y nitrato sódico de modo aislado sobre la supervivencia, masa y comportamiento (i.e. uso del hábitat y actividad) de los estadios de desarrollo larvarios de *P. perezi* en laboratorio.
- Establecer el efecto que la combinación de diferentes concentraciones de amónico, nitrito sódico y nitrato sódico ejerce sobre la supervivencia, masa y comportamiento (i.e. uso del hábitat y actividad) de los estadios de desarrollo larvarios de *P. perezi* en laboratorio.
- Realizar una aproximación al impacto de los compuestos nitrogenados en el uso del hábitat que hacen los ejemplares metamórficos de *P. perezi* en laboratorio.
- Establecer el efecto que la presencia de nitrógeno reactivo en la columna de agua tiene sobre la supervivencia, morfología y crecimiento de larvas de *P.*

perezi en condiciones con un elevado grado de naturalidad mediante ensayos realizados en mesocosmos y en localidades naturales.

- Estudiar el efecto que la presencia de nitrógeno reactivo en la columna de agua tiene sobre la aptitud de huida frente a un hipotético depredador de larvas de *P. perezi* en condiciones con un elevado grado de naturalidad mediante ensayos realizados en mesocosmos y en localidades naturales.
- Evaluar la variabilidad inter-poblacional de *P. perezi* en relación a la tolerancia a compuestos nitrogenados.
- Contrastar mediante el empleo de técnicas meta-analíticas el efecto, a escala global, de diversos compuestos químicos en diferentes parámetros de la estrategia de vida de los anfibios.

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CAPÍTULO 2

ÁREA DE ESTUDIO, DESCRIPCIÓN DE LA ESPECIE ESTUDIADA Y SINOPSIS METODOLÓGICA

ÁREA DE ESTUDIO

Se seleccionó como área de estudio la Región de Murcia para el desarrollo de los objetivos planteados en la presente Tesis Doctoral. Este territorio comprende 11.317 km^2 del sureste de la Península Ibérica. Su ubicación en el límite oriental de las Cordilleras Béticas determina que esté atravesado por una serie de alineaciones montañosas en sentido SO-NE que incluyen cotas de hasta 2.027 m.s.n.m. No obstante, la mayor parte de la superficie regional se localiza a altitudes medias-bajas, ya que casi el 25% del territorio se ubica a altitudes inferiores a 200 m.s.n.m. y el 45% entre 200 y 600 m.s.n.m., superando únicamente el 32% de la superficie regional los 600 m.s.n.m. (Sánchez *et al.*, 2002).

La orografía regional condiciona el clima del área de estudio. Así, en el NO las altas cotas presentes impiden el desplazamiento de las masas húmedas atlánticas, con lo que el volumen de las precipitaciones desciende desde las sierras noroccidentales (>600 mm anuales) hasta el mar (183 mm anuales). Por otra parte, las masas nubosas procedentes del Mar Mediterráneo ascienden rápidamente al contactar con la orografía regional, lo que produce intensas lluvias torrenciales principalmente durante otoño y primavera. Este régimen irregular de precipitaciones y las elevadas temperaturas (media anual: 16ºC-19ºC), insolación y evapotranspiración exponen a la Región de Murcia a un

clima mediterráneo semiárido, caracterizado por un amplio período de sequía en verano (Sánchez *et al.*, 2002).

El 92% de la Región (11 104 km^2) está comprendido en la cuenca hidrográfica del Río Segura (Sánchez *et al.*, 2002; Baraza, 2003), una de las más áridas de la Península Ibérica (Vidal-Abarca *et al.*, 1987) y, probablemente, de Europa (Geiger, 1973). Las localidades seleccionadas para la colección del material biológico necesario para la realización del presente trabajo y para la realización de los ensayos llevados a cabo en el campo se ubican en todos los casos en la superficie de la cuenca comprendida en la Región de Murcia (Fig. 1). Junto al Río Segura, los otros cursos de agua de caudal permanente o semipermanente presentes en la cuenca son los ríos Moratalla, Argos, Quípar, Mula y Guadalentín, afluentes todos ellos del Río Segura por la margen derecha (Baraza, 2003). Adicionalmente, la red hidrográfica del área de estudio está caracterizada por la presencia de ramblas, cursos de agua definidos por la variabilidad de la presencia de agua, tanto espacial como temporalmente (Baraza, 2003). Al margen de los mencionados sistemas de drenaje, la superficie de la Región incluye 82 humedales naturales correspondientes a diferentes tipologías que incluyen desde fuentes y manantiales hasta lagunas costeras (Ballester, 2003). Sin embargo, en comparación con el número de humedales naturales, los de origen artificial son mucho más numerosos. Así, fruto de la modificación de la red natural de drenaje, en el área de estudio están presentes 14 embalses junto a, al menos, 2000 balsas en el conjunto de áreas regables para su aprovechamiento agrícola (Ballester, 2003). Estos cuerpos de agua representan el único hábitat acuático disponible en gran parte de la Región de Murcia. Como consecuencia, su frecuencia de utilización como hábitat reproductor por parte de los anfibios es elevada para la mayor parte de las especies presentes en el área de estudio, a pesar de que la utilización agrícola de estas masas de agua expone a los

anfibios a factores de amenaza tan diversos como la presencia de vertidos o la modificación del medio acuático (Egea-Serrano *et al.*, 2005a).

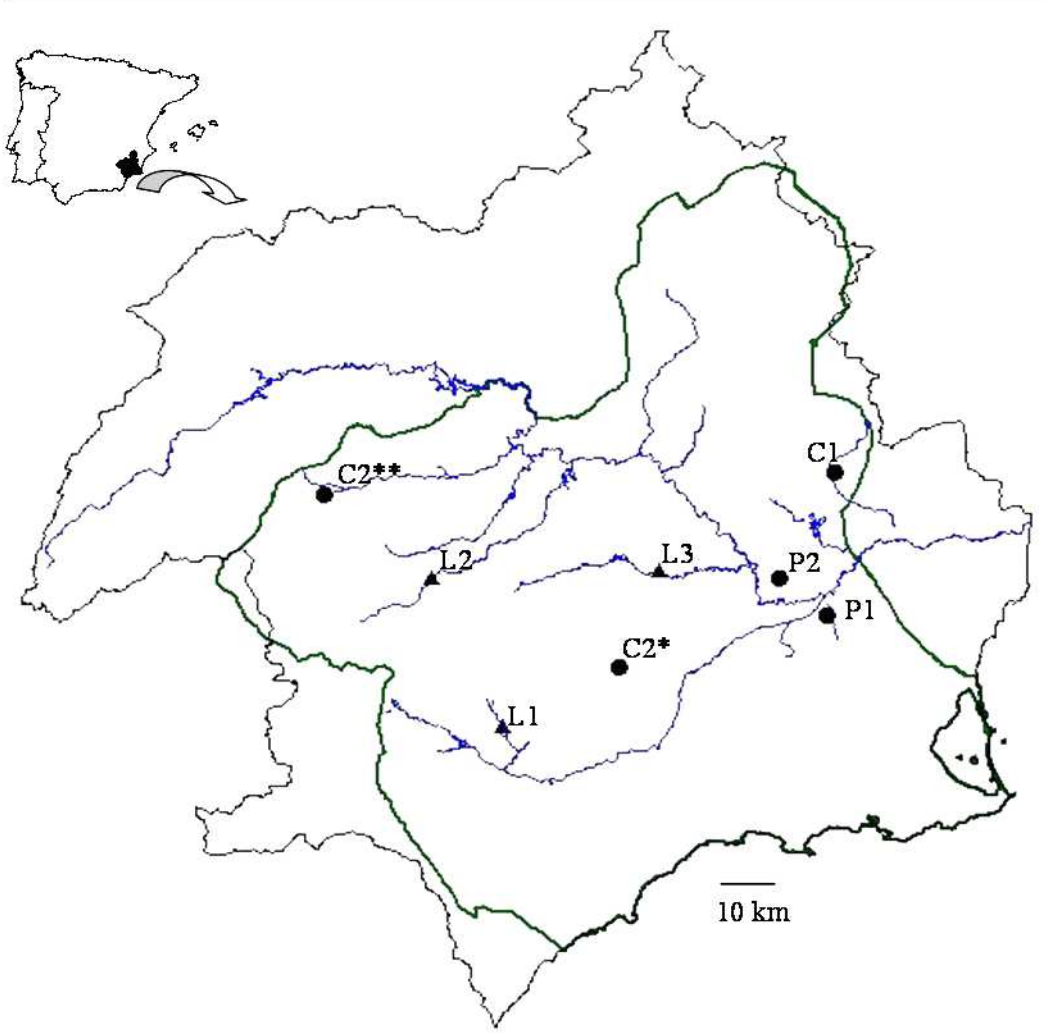

Fig. 1. Situación en la cuenca del Río Segura de las poblaciones de *Pelophylax perezi* muestreadas en el presente estudio (●) y de las localidades seleccionadas para el desarrollo de la experimentación en localidades naturales (▲). Resaltado en verde se indica el contorno de la Región de Murcia. Los principales cursos de agua de la cuenca se representan en azul. C2*: población considerada para la experimentación en localidades naturales; C2**: población considerada para la experimentación en laboratorio y localidad seleccionada para la exposición en localidades naturales.

Las actividades agrícolas en el área de estudio representan un incremento casi del 300% de la superficie destinada a los cultivos de regadío en el siglo XX (Baraza, 2003). Esta expansión ha supuesto la alteración de numerosos ecosistemas, la desaparición de numerosos hábitats, tanto terrestres como acuáticos, la regresión de las explotaciones agrícolas tradicionales y la pérdida de heterogeneidad en los agrosistemas (Baraza, 2003). Por otra parte, la ocupación de parte del litoral, el desarrollo de los principales núcleos urbanos y la ocupación de las mejores tierras de regadío ha permitido una importante expansión urbana y suburbana (Baraza, 2003). Estas transformaciones en los usos del suelo han supuesto un impacto negativo sobre la calidad de los cuerpos de agua tanto lóticos como lénticos presentes en el área de estudio, los cuales reciben vertidos depurados deficientemente y la escorrentía de los campos agrícolas (Baraza, 2003) y muestran una tendencia a la eutrofización (Ballester, 2003). Como consecuencia, el área de estudio representa un territorio óptimo para abordar el estudio del impacto de las actividades antrópicas en general, y de la contaminación en particular, sobre la vida silvestre.

La Región de Murcia se extiende, desde un punto de vista biogeográfico, por la Región Mediterránea (Sánchez *et al.*, 2002). Actualmente, la vegetación natural está dominada por matorrales y pastizales. Las masas boscosas están formadas principalmente por pinares, los cuales se restringen al 15% de la superficie regional debido a los incendios forestales y a las condiciones climáticas áridas (Sánchez *et al.*, 2002). La extensión de la superficie ocupada por carrascales está en recesión y la presencia de sabinares y de especies caducifolias es testimonial (Sánchez *et al.*, 2002).

Por lo que respecta a la fauna, la Región de Murcia está caracterizada por un elevado número de especies de invertebrados, tanto artrópodos como no artrópodos, destacables por su importancia conservacionista (Baraza, 2003). Asimismo, entre los

vertebrados, la gran heterogeneidad ambiental ha originado una gran riqueza específica, destacando taxa exclusivamente ibéricos, mediterráneos occidentales o presentes también el norte de África (e.g. *Testudo graeca* Linnaeus, 1758, *Squalius pyrenaicus* (Günther, 1868), *Capra pyrenaica* Schinz, 1838) (Baraza, 2003). En relación a los anfibios, la Región de Murcia presenta una notable pobreza, debido probablemente a la lejanía respecto a los principales centros de especiación en la Península y a la aridez del entorno (Mateo, 2002). No obstante, la Región de Murcia, junto al resto del sureste peninsular ha sido descrita como uno de los territorios más importantes en la Región Mediterránea debido a la riqueza y/o grado de endemicidad de las especies de anfibios presentes (Borkin, 1999). El número de especies detectadas asciende a nueve, si bien la presencia de *Hyla meridionalis* Boettger, 1874 y de *Pleurodeles waltl* Michahelles, 1830 ha sido descrita con anterioridad (Hernández-Gil *et al.*, 1993; Egea-Serrano *et al.*, 2005a, b). La mayor parte de ellas, excepto *Bufo calamita* (Laurenti, 1768) y *Pelophylax perezi* (Seoane, 1885), son mediana o altamente vulnerables debido a sus características biológicas y ecológicas (Egea-Serrano *et al.*, 2006), por lo que han sido asignadas a categorías de amenaza elevadas (Egea-Serrano *et al.*, 2007). No obstante, la menor vulnerabilidad descrita para *B. calamita* y *P. perezi* y su amplia distribución en la Región de Murcia no implica que no estén amenazadas en este territorio ya que, aunque no existen datos sobre las tendencias demográficas de sus poblaciones, sí se ha constatado una significativa disminución del área de distribución regional en los últimos años, al menos en el caso de *B. calamita* (Egea-Serrano *et al.*, 2007). Así, las poblaciones de estas dos especies de anuros podrían verse afectadas, dada su capacidad para ocupar ambientes sometidos a una importante presión antrópica, por la degradación del hábitat, introducción de especies exóticas y contaminación del hábitat, todos los cuales se han reconocido como importantes factores de regresión de poblaciones de

anfibios en todo el mundo (Alford & Richards, 1999; Blaustein & Kiesecker, 2002; Gardner, 2001; Collins & Storfer, 2003; Semlitsch, 2003; Beebee & Griffiths, 2005).

DESCRIPCIÓN DE LA ESPECIE ESTUDIADA

Reino: Animalia Linnaeus, 1758

Phylum: Chordata Bateson, 1885

Clase: Amphibia Linnaeus, 1758

Orden: Anura Rafinesque, 1815

Familia: Ranidae Rafinesque, 1814

Género: *Pelophylax* Fitzinger, 1843

Especie: *Pelophylax perezi* (Seoane, 1885)

Entre las especies de anfibios presentes en el área de estudio, se ha seleccionado como especie modelo para la realización del presente trabajo *P. perezi* como consecuencia de la facilidad para la localización de poblaciones viables y la colección de frezas debido a que: 1) la especie habita preferentemente cuerpos de agua permanentes (Richter-Boix *et al.*, 2007); 2) el inicio de su época reproductora no está relacionado con las precipitaciones (Díaz-Paniagua, 1992); y, 3) está ampliamente distribuida por la Península Ibérica (Llorente *et al.*, 2002), incluido el área de estudio (Egea-Serrano *et al.*, 2005a, b). En relación a esta última característica, se ha descrito que especies de anfibios muy abundantes o ampliamente distribuidas pueden no ser modelos óptimos debido a una hipotética tolerancia a la contaminación (Marco, 2002), a pesar de ser ampliamente usadas en estudios ecotoxicológicos (especialmente en el hemisferio norte) (Schiesari *et al.*, 2007). No obstante, ha sido reconocida la necesidad del estudio de

especies comunes por su importancia en el funcionamiento de los ecosistemas (Gaston & Fuller, 2007). Aunque la decadencia de estas especies sea proporcionalmente reducida, puede implicar una importante pérdida de abundancia y/o biomasa, lo que, dada la relevancia de los anfibios en la cadena trófica (Blaustein *et al.*, 1994), repercutiría negativamente en los ecosistemas. Esta consideración, unida a la necesidad ética de evitar la pérdida de biodiversidad, justifica la profundización en el estudio del impacto que la contaminación produce sobre especies de anfibios comunes.

P. perezi se distribuye ampliamente por la Península Ibérica y el sur de Francia, correspondiendo las discontinuidades observadas a nivel peninsular a una prospección deficiente en lugar de a una auténtica ausencia de la especie (Llorente *et al.*, 2002). Ha sido introducida en las Islas Baleares, Islas Canarias, Madeira e Islas Azores (Malkmus, 1997; Corti *et al.*, 1999; Llorente *et al.*, 2002). Presenta un tamaño máximo de 85 mm en machos y 110 mm en hembras (Salvador & García-París, 2001). Está caracterizada por presentar tímpano patente, pupila horizontal, pliegue glandular dorsolateral moderadamente desarrollado y conspicuos tubérculos subarticulares en los dedos de las cuatro extremidades (García-París *et al.*, 2004). Las extremidades posteriores están unidas por membranas interdigitales ampliamente desarrolladas (García-París *et al.*, 2004). Tanto la piel ventral como la dorsal son lisas, presentando una coloración blanca o jaspeada de negro en el primer caso y usualmente verde con manchas negras en el segundo (García-París *et al.*, 2004) (Lámina 1). Las larvas presentan la morfología generalista de las especies adaptadas a vivir en charcas (Duellman & Trueb, 1994), lo que las incluiría entre las especies adaptadas a vivir en el fondo de los cuerpos de agua (Díaz-Paniagua, 1985). Los estadios de desarrollo larvario presentan un cuerpo algo deprimido que exhibe el espiráculo en el costado izquierdo y el ano en el derecho, en posición posterior (García-París *et al.*, 2004). La cola es larga, con una cresta caudal

más desarrollada dorsal que ventralmente que termina en ángulo agudo (García-París *et al.*, 2004). Normalmente los estadios larvarios de la especie estudiada alcanzan longitudes de hasta 6 cm, aunque se han descrito individuos de 9 cm (Llorente *et al.*, 1995). Respecto a los dentículos córneos labiales, se han descrito 19 morfotipos orales, si bien el más común es el que presenta la primera fila superior completa y la segunda dividida por un amplio espacio, la primera inferior dividida por un amplio espacio, la segunda completa y la tercera completa pero corta (Llorente *et al.*, 1995). La coloración dorsal de las larvas son verdes con manchas oscuras, mientras que ventralmente son blancas con reflejos metálicos o nacarados (Llorente *et al.*, 1995) (Lámina 1).

El período de actividad de *P. perezi* se extiende durante la mayor parte del año, aunque reduce su actividad en invierno (Malkmus, 1987; Pollo *et al.*, 1998). Especie estrictamente acuática, rara vez se separa más de 5 m del agua (Lizana *et al.*, 1989) aunque presenta cierta capacidad de dispersión por tierra firme (Malkmus, 1987; Diaz-Paniagua & Rivas, 1987). Ocupa gran diversidad de hábitat acuáticos, tanto lóticos como lénticos de origen natural y artificial (e.g. Egea-Serrano *et al.*, 2005c). Aunque puede estar presente tanto en cuerpos de agua permanentes como temporales, no se trata de una especie característica de ambientes temporales (Richter-Boix *et al.*, 2007). Altitudinalmente ocupa cuerpos de agua situados desde el nivel del mar (Egea-Serrano *et al.*, 2005d) hasta los 2.380 m.s.n.m. (Fernández-Cardenete *et al.*, 2000), si bien habita preferentemente localidades ubicadas a altitud media (Egea-Serrano *et al.*, 2005d; García & Lizana, 2007). El carácter eminentemente anfibio de la especie estudiada tras la finalización de la metamorfosis hace que use el medio acuático como refugio frente a depredadores (Martín *et al.*, 2005), entre los que se encuentran reptiles, aves y mamíferos (Rey *et al.*, 1994; Santos *et al.*, 2000; Clavero *et al.*, 2005), como fuente de alimento, a pesar de alimentarse fundamentalmente de presas terrestres (Hódar *et al.*,

1990) y como hábitat reproductor (Egea-Serrano *et al.*, 2005c). Por lo que respecta a este último aspecto, *P. perezi* utiliza una gran diversidad de cuerpos de agua, tanto naturales como artificiales (Egea-Serrano *et al.*, 2005c), seleccionando positivamente ambientes permanentes (Richter-Boix *et al.*, 2007), excepto bebederos y balsas de riego (Egea-Serrano *et al.*, 2005c), con abundante vegetación de ribera (Egea-Serrano *et al.*, 2005c).

El período reproductor de la especie estudiada es tardío en relación a las restantes especies de anfibios presentes en la Península Ibérica (Salvador & Carrascal, 1990; Díaz-Paniagua, 1992; Egea-Serrano *et al.*, 2005e), estando correlacionado positivamente con la temperatura ambiental (Richter-Boix *et al.*, 2006) y siendo independiente de las precipitaciones (Díaz-Paniagua-Paniagua, 1992). Se ha detectado la presencia de frezas en la Península Ibérica desde abril hasta julio, si bien existen evidencias de que la especie puede reproducirse hasta agosto/ septiembre (Egea-Serrano *et al.*¸ 2005e). Durante este período, es depositado un número medio de 2.309 huevos (Hotz *et al.*, 1994), pudiendo algunas hembras desovar dos o tres veces durante la época reproductora (Hotz *et al.*, 1994). Tras un desarrollo embrionario de 5-8 días (García-París, 2000), las larvas completan usualmente la metamorfosis al cabo de dos meses, si bien la duración del desarrollo larvario es variable (Díaz-Paniagua, 1986). Los ejemplares metamórficos emergen en verano y otoño (Pollo *et al.*, 1998; Egea-Serrano *et al.*, 2005e), aunque las larvas hibernantes pueden completar su desarrollo durante la primavera siguiente (Álvarez *et al.*, 1991; Richter-Boix *et al.*¸ 2006). Los ejemplares metamórficos alcanzan la madurez sexual usualmente durante el primer o segundo año de vida (Docampo & Milagrosa-Vega, 1991; Esteban *et al.*, 1996), presentando una longevidad máxima de 5 años en el caso de los machos y de 6 en el caso de las hembras (Patón *et al.*, 1991).

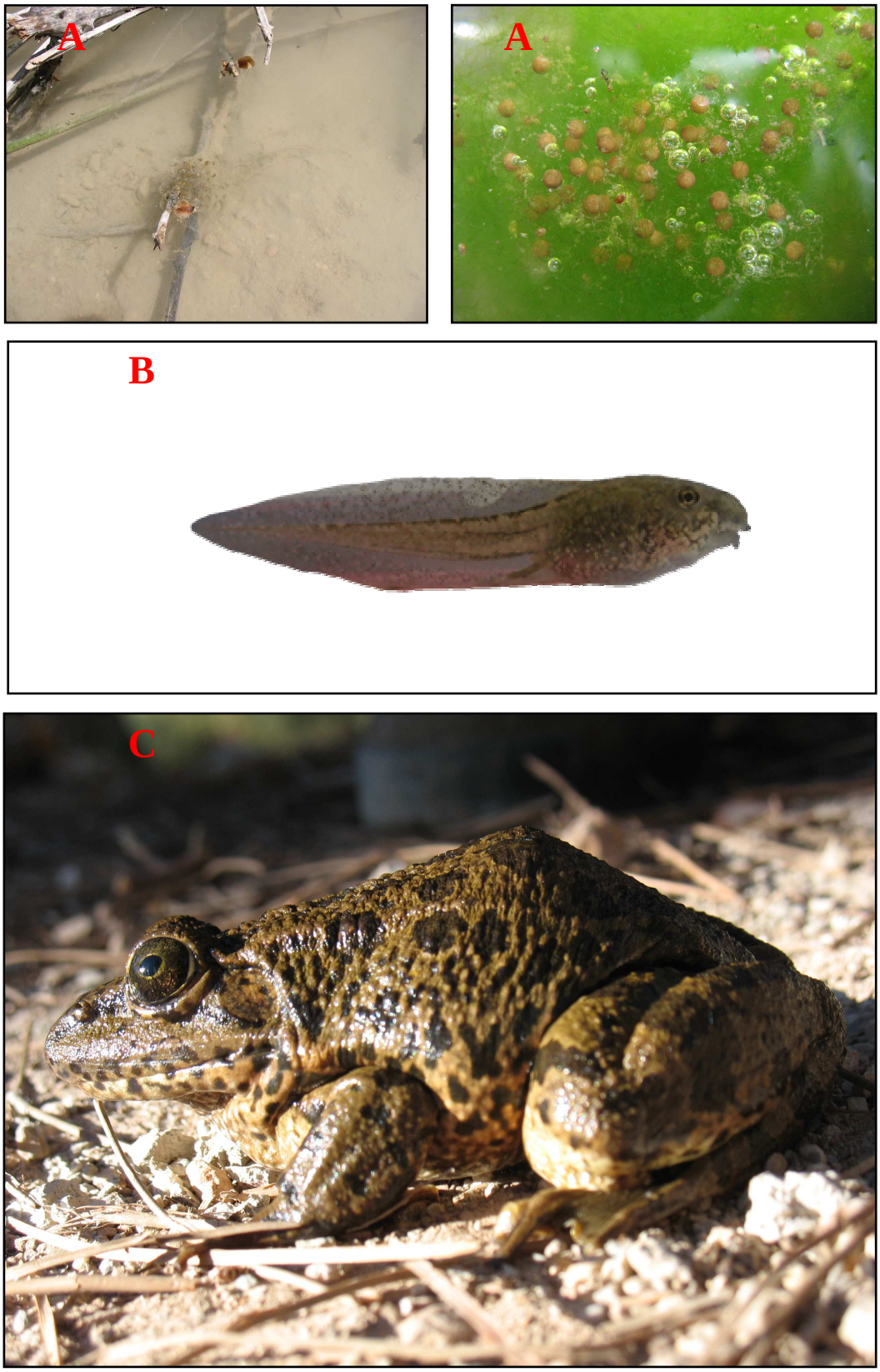

Lámina 1. Diferentes estadios de desarrollo de *Pelophylax perezi*. A: Embriones; B: Larva; C: adulto.

Al igual que muchas otras especies de anfibios, *P. perezi* está amenazada por la destrucción del hábitat (Malkmus, 2004; Galán, 1999), la presencia de especies exóticas (Arano *et al.*, 1995; Galán, 1997; Martínez-Solano *et al.*, 2003) y, en menor medida, la captura directa de ejemplares (Galán, 1999). Asimismo, la contaminación del hábitat representa un importante factor de amenaza, ya que la exposición a pesticidas, vertidos mineros, radiación ultravioleta o compuestos nitrogenados, así como a la interacción de algunos de estos contaminantes, afecta significativamente a la supervivencia, desarrollo, comportamiento de la especie e incrementa la concentración de metales pesados y pesticidas en sus tejidos (Rico *et al.*, 1987; Honrubia *et al.*, 1993; Álvarez *et al.*, 1995; Tejedo & Reques, 2003; Pastor *et al.*, 2004; Macías *et al.*, 2007; Shinn *et al.*, 2008).

Pelophylax perezi **en la Región de Murcia**

La distribución de *P. perezi* se extiende por casi toda la superficie regional (Egea-Serrano *et al.*, 2005a, b; Torralva *et al.*, 2005). Altitudinalmente se encuentra de forma casi continua desde el nivel del mar hasta casi 1600 m.s.n.m. (Egea-Serrano *et al.*, 2005d).

Su presencia y reproducción ha sido constatada en las diferentes tipologías de cuerpos de agua presentes en la Región de Murcia (Egea-Serrano *et al.*, 2005a, c; Torralva *et al.*, 2005). Aunque en la superficie regional las balsas de riego representan los ambientes utilizados más frecuentemente por la especie (Egea-Serrano *et al.*, 2005a; Torralva *et al.*, 2005), ésta muestra una marcada selección positiva hacia bebederos, charcas y arroyos (Egea-Serrano *et al.*, 2005c). A escala de microhábitat, selecciona cuerpos de agua con una cobertura de vegetación de ribera moderada o alta (Egea-Serrano *et al.*, 2005c).

El período reproductor de algunas poblaciones de *P. perezi* en la Región de Murcia es dilatado, extendiéndose desde abril hasta julio (Egea-Serrano *et al.*, 2005e). La emergencia de los ejemplares metamórficos tiene lugar desde julio hasta octubre (Egea-Serrano *et al.*¸ 2005e). Las charcas y bebederos destacan por la densidad larvaria relativa de la especie estudiada (Egea-Serrano *et al.*, 2005e).

P. perezi es una de las especies menos vulnerables a la extinción de entre las presentes en la Región de Murcia (Egea-Serrano *et al.*, 2006), hecho que concuerda con la evaluación obtenida tras la aplicación de los criterios UICN a nivel regional (Egea-Serrano *et al.*, 2007). Sin embargo, esta escasa vulnerabilidad no quiere decir que la especie no esté amenazada, ya que el análisis descriptivo de los factores de amenaza a los que están expuestos los cuerpos de agua presentes en la Región de Murcia evidenció que la modificación del medio acuático y la presencia de vertidos son las principales amenazas a las que está expuesta *P. perezi* a escala regional (Egea-Serrano *et al.*, 2005a; Torralva *et al.*, 2005). No obstante, información detallada sobre el efecto que diferentes agentes estresantes puede tener sobre esta especie en el área de estudio es casi inexistente (pero ver Egea-Serrano *et al.*, 2008, 2009a, b).

SINOPSIS METODOLÓGICA

La metodología empleada en los diferentes estudios realizados se describe con detalle en cada uno de los capítulos correspondientes. Sin embargo, con la finalidad de facilitar la comprensión de la presente Tesis Doctoral en su conjunto, se describen brevemente a continuación los diferentes aspectos metodológicos contemplados durante su realización. Los números arábigos destacados en negrita señalan los capítulos donde las diferentes metodologías descritas en el texto han sido aplicadas.

Colección e incubación de embriones.- Se colectaron cinco masas de huevos de *P. perezi* en una (**3**, **6**), tres (**7**, **8**) o cuatro (**4**, **5**) poblaciones silvestres localizadas en el área de estudio (ver figura 1 en capítulo **2**). Los huevos fueron trasladados al laboratorio, donde se incubaron en acuarios de cristal con 12 l de agua de grifo desclorada. Los diferentes experimentos se iniciaron una vez las larvas alcanzaron los estadios de desarrollo de Gosner 25 (**3**, **4**, **5**, **7**, **8**) o 46 (**6**) (Gosner, 1960).

Diseño experimental.- Los experimentos fueron llevados a cabo en condiciones de laboratorio (**3**, **4**, **5**, **6**), mesocosmos (**7**, **8**) o en localidades naturales expuestas a diferentes grados de contaminación (ver figura 1 en capítulo **2**) (**7**, **8**). En el caso de los experimentos realizados en laboratorio o mesocosmos, los individuos experimentales se expusieron a 4 (**6**, **7**, **8**), 11 (**4**, **5**) o 14 (**3**) tratamientos que incluyeron tanto un único compuesto nitrogenado (NH_4Cl, $NaNO_2$, $NaNO_3$) como la combinación de varios de ellos. Las larvas se dispusieron en grupos de 6 (**3**) o de manera aislada (**4**, **5**, **7**, **8**) en contenedores plásticos de 1 l (**4**, **5, 7, 8**) o 1,5 l de capacidad (**7**, **8**). Los individuos juveniles fueron asimismo expuestos de forma individual a los tratamientos correspondientes en recipientes plásticos de 1 l de capacidad (**6**). Las larvas fueron alimentadas *ad libitum* con pienso para perros en el caso de los estudios realizados en laboratorio (**3**, **4**, **5**), mientras que en los mesocosmos o corrales (**7**, **8**) sólo se les proporcionó alimento (pienso para perros) una única vez al inicio del experimento. Los ejemplares juveniles fueron alimentados *ad libitum* con moscas de la fruta (**6**). La duración de los experimentos fue de 3 (**3**), 4 (**3**), 14 (**6**) o 21 días (**4**, **5**, **7**, **8**). En el caso de los estudios en laboratorio (**3**, **4**, **5**, **6**), las unidades experimentales fueron dispuestas al azar en las diferentes baldas de una estantería, mientras que en los estudios de

mesocosmos y campo (**7**, **8**) las larvas procedentes de diferentes poblaciones de origen (individualizadas en los correspondientes recipientes) se agruparon en diferentes mesocosmos y corrales, respectivamente, según un diseño *split-plot*, el cual asume que las diferentes categorías de un factor dado (e.g. tratamiento o localidad) no están replicadas en todas las categorías de un segundo factor (e.g. mesocosmo o corral) (Quinn & Keough, 2002).

Por otra parte, mediante técnicas meta-analíticas se evaluó el impacto de la contaminación química sobre los anfibios como grupo (**9**). Para ello se realizó una extensa búsqueda bibliográfica, obteniéndose para cada tratamiento considerado en las publicaciones seleccionadas los valores correspondientes a media, desviación típica (SD) y número de casos (n).

Variables estudiadas.- Las variables estudiadas fueron: supervivencia (**3**, **4**, **7**), consumo de alimento (**4**), masa al final del experimento (**4**, **7**), número de censos que los individuos experimentales se detectaron inactivos (**5**), número de censos que los individuos experimentales se detectaron sobre el fondo de los contenedores (**5**), número de veces que los animales se detectaron en contacto con el medio acuático (**6**), crecimiento (**7**), morfología (i.e. longitud del cuerpo y de la cola, altura del cuerpo, de la porción muscular de la cola y de la cola incluyendo la cresta, anchura del cuerpo y anchura de la base de la cola) (**7**), velocidad de natación y distancia recorrida (**8**).

En el caso del meta-análisis (**9**), se estudió la influencia de la contaminación sobre los anfibios como grupo atendiendo a las variables: supervivencia, tiempo hasta la eclosión y metamorfosis, longitud, masa, tasa de malformaciones.

Análisis estadísticos.- Se analizaron los datos obtenidos mediante ANCOVAs (**6**), ANCOVAs de medidas repetidas (**6**), ANOVAs de medidas repetidas (**3**) y Modelos Lineares Generales (GLM) (**4**, **5**, **7**, **8**) para establecer la existencia de diferencias significativas entre los valores medios de las variables estudiadas para cada tratamiento experimental. Cuando la variable dependiente fue de naturaleza binaria, el análisis de la existencia de diferencias entre los valores medios para cada tratamiento se realizó mediante Modelos Lineares Generalizados (GLZ) para datos binarios (**4**, **7**). Adicionalmente, se emplearon Análisis de Regresión Probit para el establecimiento de los valores LC_{50} (concentración letal media) correspondientes a diferentes compuestos nitrogenados (**3**) y mediante Análisis de Regresión Lineal Múltiple se determinó la influencia de la morfología o crecimiento larvario sobre la aptitud de huida de las larvas de la especie estudiada (**7**). Se consideraron como factores las siguientes variables: ambiente (**4**, **5**), población (**4**, **5**, **7**, **8**), tratamiento o localidad de destino (**3**, **4**, **5**, **6**, **7**, **8**), y las respectivas interacciones.

El impacto global de la contaminación química sobre los anfibios se determinó mediante el cálculo del estadístico d^+ de Hedge, el cual proporciona una medida de la magnitud del efecto del tratamiento considerado en cada estudio (Rosenberg *et al.*, 2000). Calculado el estadístico d^+ para cada estudio incluido en la matriz de datos a analizar mediante meta-análisis, se estableció el impacto global medio mediante Modelos de Efectos Aleatorios y se analizó la existencia de diferencias significativas entre las categorías de grupos definidos *a priori* (familia, estadio de desarrollo, condiciones experimentales, tipo de contaminante) mediante Modelos de Efectos Mixtos (**9**).

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BLOQUE II

ANÁLISIS DE LOS EFECTOS DE LOS COMPUESTOS NITROGENADOS EN *PELOPHYLAX PEREZI* EN EXPERIMENTOS DE LABORATORIO

CAPÍTULO 3

ESTIMATING MEAN LETHAL CONCENTRATIONS OF THREE NITROGENOUS COMPOUNDS FOR THE IBERIAN WATERFROG, *PELOPHYLAX PEREZI* (SEOANE, 1885), LARVAE

Abstract: The sensitivity of *Pelophylax perezi* larvae from a natural population located in the Segura River basin (southeastern Spain) to three nitrogenous compounds (NH_4Cl, $NaNO_2$ and $NaNO_3$) was analysed. Larval mortality was significantly increased by raising concentrations and exposure time to these compounds. The LC_{50} values decreased as time went on for all the nitrogenous compounds. LC_{50} values obtained for NO_2^- and NO_3^- ions are in all cases higher than the peak concentrations found in the environment but this is not the case for NH_4^+ ion, for which LC_{50} values obtained are lower than the concentrations found in the field. This may threaten populations of *P. perezi*, species highly sensitive to NH_4^+ pollution, which could be exposed to lethal concentrations of the NH_4^+ ion and, therefore, be potentially suffering a decline as a consequence of eutrophication.

Key words: Lethal concentrations, nitrogenous compounds, *Pelophylax perezi*, tadpoles

INTRODUCTION

Habitat destruction and degradation have been described as one of the major threats faced by amphibians at present (Stuart *et al.*, 2004). Such degradation may be a consequence of habitat fragmentation, the alteration and suppression of natural ecosystem processes, introduction of exotic species and the presence of pollutants (Dodd & Smith, 2003). Among the latter, fertilizers, which have been used intensively in last decades, are considered to have a potential impact on amphibian populations because they concentrate in waterbodies located within agricultural areas (Berger, 1987, 1989; Hamer *et al.*, 2004, but see Massal *et al.*, 2007). Some experimental approaches have revealed their negative effect on amphibian survival and life history traits (e.g. Ortiz *et al.*, 2004). However, the effects that contamination may have on amphibian species are not well known, and so it becomes a matter of conservation priority to determine the level of potential tolerance to fertilizers that amphibian species may withstand by conducting standardized toxicological experiments (Marco & Ortiz-Santaliestra, in press).

LC_{50} (mean lethal concentration) assays are the most common tests used to determine the sensitivity of a species to a pollutant (Bridges & Semlitsch, 2001). These assays consist of determining, by using different concentrations of a chemical, the concentration at which 50% of a test population dies. As a consequence, naturally occurring concentrations of such chemical in the environment that equal or exceed the LC_{50} value may lead to population extinction. Additionally, and although the concentrations necessary to induce direct mortality may be higher than actual concentrations in the environment (Boone & Bridges, 2003), LC_{50} values can be used to establish sublethal levels of a pollutant and to study their effects (Bridges, 1999).

With regard to fertilizers, LC_{50} assays have been performed for different nitrogenous compounds in several species of amphibians (see review Marco & Ortiz-Santaliestra, in press). One of the major conclusions of these studies is the great inter- and intraspecific variation described in relation to the LC_{50} value of nitrite and nitrate (Hecnar, 1995; Marco *et al.*, 1999; Shinn *et al.*, 2008). This highlights the importance of determining the actual sensitivity to nitrogenous compounds of a broad range of species to determine their potential impact on amphibian populations and communities.

The aim of this study was to analyze the sensitivity to nitrogenous pollutants of a larval population of *Pelophylax perezi* by determining the LC_{50} values for ammonium, nitrite and nitrate compounds. This species is an European waterfrog whose distribution ranges through the Iberian Peninsula and southern France (Llorente & Arano, 1997). It mainly inhabits permanent waterbodies (Díaz-Paniagua, 1990), especially those showing high riparian vegetation cover (Egea-Serrano *et al.*, 2005). Because these permanent waterbodies may hold high concentrations of nitrogenous compounds as a result of farming practices and urban sewage (one of the main nitrogen sources in the environment, e.g. Ritter & Bergstrom, 2001), aquatic stages of *P. perezi* may potentially be threatened by nitrogen pollution, as the results showed by Macías *et al.* (2007) suggest concerning nitrite. This hypothesis contradicts the tolerance to pollution mentioned for this species in previous publications (Llorente *et al.*, 2002). So, a great effort needs to be made to accurately assess the actual sensitivity of larvae of *P. perezi* to nitrogenous compounds.

MATERIAL AND METHODS

We sampled five different egg masses obtained from a natural *P. perezi* population located in the Segura River basin (southeastern Spain, U.T.M. 30SWH, 1197.92 m.a.s.l.), not exposed to eutrophication (e.g. <2.1 mg NO_3^-/l, unpublished data). The samples belonging to the five clutches were pooled to increase the probability of analysing a representative sample of the genetic variation within the population. Embryos were reared in the laboratory in 12 l glass aquaria, at roughly 25ºC, in aerated dechlorinated tap water. When they reached Gosner developmental stage 25 (Gosner, 1960) (total length: 10.79 mm ± 0.18 mm, n= 230), they were transferred to clear, food-quality, 1 l plastic beakers containing 0.5 l of test solution. Ammonium, nitrite and nitrate solutions were prepared from NH_4Cl, $NaNO_2$ and $NaNO_3$, respectively. Treatments consisted of the following nominal increasing concentrations: 0, 15, 30, 60, 120 mg NH_4Cl/l; 0, 500, 1000, 5000, 10 000, 20 000 mg $NaNO_2$/l; 0, 2000, 5000, 10 000, 20 000 mg $NaNO_3$/l. Each beaker, containing six haphazardly chosen larvae, was randomly assigned to one of the previous treatments. Each treatment was replicated three times in the case of NH_4Cl and twice for $NaNO_2$ and $NaNO_3$ due to a shortage of tadpoles.

The experiment consisted of static renewal tests (Stephen, 1975). Water treatments were renewed daily and dead animals were removed from the experimental units to avoid oxygen depletion. Beakers were loosely closed to avoid water evaporization and ammonium volatization. Tadpoles were fed with dried dog chow, of which a food pellet (250-350 mg) was added each day. Tadpoles were observed each 12 h over a 96 h period for mortality in the case of NH_4Cl treatments. As regards $NaNO_2$ and $NaNO_3$, the observation period was limited to 72 h.

To assess the effect of the concentrations used in this study (independent variables) on larval mortality (dependent variable: number of dead tadpoles in each beaker), repeated measures ANOVAs were performed separately for each nitrogenous compound. Probit analysis was used to determine LC_{50} for NH_4^+, NO_2^- and NO_3^- ions during 24-h, 48-h, 72-h and 96-h exposure. Data were transformed logarithmically. Statistical analyses were performed using SPSS® statistical package v. 11.0 and a significant level of 5% was selected.

RESULTS

Table 1 shows the number of tadpoles that died as a consequence of exposure to the different treatments. No mortality was recorded for the control treatment. For all three nitrogenous compounds, both increasing concentrations and exposure times increased larval mortality ($p < 0.0001$, Table 1). Nevertheless, for NH_4Cl no larval mortality was recorded for concentrations lower than 60 mg NH_4Cl/l. The significant concentration x time interactions ($p < 0.001$, Table 1) revealed that the effects produced by the concentrations used for the three nitrogenous compounds differed between observation times. So, the highest concentrations produced the highest larval mortality earlier than the remaining treatments.

The results of the probit analyses are shown in Table 2. LC_{50} values decreased at each observation period for all the nitrogenous compounds. Figure 1 shows dose-response curves for larvae of *P. perezi* exposed to NH_4^+, NO_2^- and NO_3^- ions. In the case of NH_4^+ and NO_3^-, the concentration needed to kill 100% of the larvae exposed decreased with time. However, in the case of NO_2^-, the concentration needed to kill

100% after 48 hours of exposure was lower than that observed after 24 and 72 hours of exposure.

Table 1. Accumulated number of dead *Pelophylax perezi* tadpoles occurring at 24, 48, 72 and 96 hours after exposure. Summary statistics for repeated measures ANOVAs for the effects of the three nitrogenous compounds tested on larval mortality over time are shown.

Nitrogenous compound	Concentration (mg/l)	n	Time			
			24h	48h	72h	96h
NH_4Cl	0	18	0	0	0	0
Concentration: $F_{4,10}$= 491.727, p < 0.0001	15	18	0	0	0	0
Time $F_{3,30}$= 34.099, p < 0.0001	30	18	0	0	0	0
Concentration x Time:	60	18	0	0	2	12
$F_{12,30}$= 22.603, p < 0.0001	120	18	9	16	18	18
$NaNO_2$	0	12	0	0	0	-
Concentration: $F_{5,6}$= 58.565, p < 0.0001	500	12	0	0	2	-
Time: $F_{2,12}$= 85.015, p < 0.0001	1000	12	1	1	3	-
Concentration x Time:	5000	12	10	10	12	-
$F_{10,12}$= 29.741, p < 0.0001	10 000	12	12	12	12	-
	20 000	12	12	12	12	-
$NaNO_3$	0	12	0	0	0	-
Concentration: $F_{4,5}$= 46.872, p < 0.0001	2000	12	0	1	1	-
Time: $F_{2,10}$= 25,864, p < 0.0001	5000	12	0	1	11	-
Concentration x Time:	10 000	12	1	11	12	-
$F_{8,10}$= 9.761, p < 0.001	20 000	12	12	12	12	-

Table 2. Results of the probit analysis for ions NH_4^+, NO_2^- and NO_3^- at 24, 48, 72 and 96 hours after exposure. SE: standard error. LC_{50}: mean lethal concentration. CI: confidence interval.

Nitrogenous compound	Time (h)	Probability of mortality (PM)	SE on slope	LC_{50}	
				LC_{50} (mg/l)	95% CI
NH_4^+	24	PM= -7.10 + 4.23 $\log_{10}([NH_4^+]+1)$	1.51	45.39	34.96-106.12
	48	PM= -10.56 + 7.06 $\log_{10}([NH_4^+]+1)$	1.78	31.37	25.79-38.24
	72	PM= -10.47 + 7.34 $\log_{10}([NH_4^+]+1)$	1.80	26.69	22.24-32.51
	96	PM= -10.31 + 8.02 $\log_{10}([NH_4^+]+1)$	2.15	19.27	16.03-22.90
NO_2^-	24	PM= -12.03 + 3.72 $\log_{10}([NO_2^-]+1)$	0.80	1697.60	1077.15-2552.54
	48	PM= -17.25 + 5.56 $\log_{10}([NO_2^-]+1)$	1.78	1270.90	848.90-3038.71
	72	PM= -7.92 + 2.67 $\log_{10}([NO_2^-]+1)$	0.62	914.59	579.60-1526.53
	96	-	-	-	-
NO_3^-	24	PM= -18.23 + 4.59 $\log_{10}([NO_3^-]+1)$	1.38	9440.12	6967.97-13959.02
	48	PM= -16.41 + 4.48 $\log_{10}([NO_3^-]+1)$	1.04	4611.02	2299.23-9404.95
	72	PM= -17.98 + 5.33 $\log_{10}([NO_3^-]+1)$	1.46	2381.53	1697.45-3230.75
	96	-	-	-	-

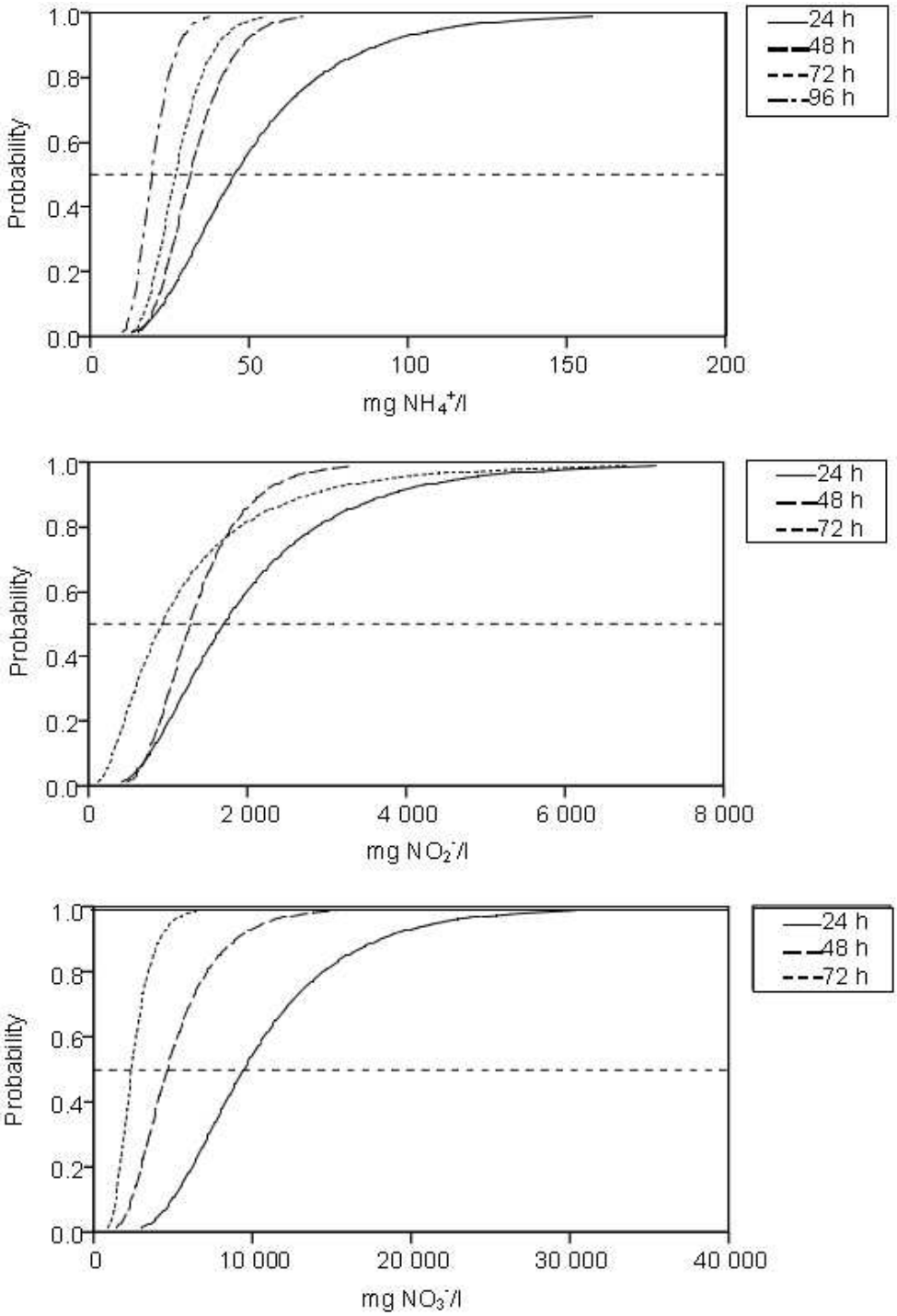

Fig. 1. Dose-response curves for mortality of *Pelophylax perezi* larvae exposed to NH_4^+, NO_2^- and NO_3^-. The LC_{50} value for each time interval is graphically represented by the dotted line in each plot.

DISCUSSION

The results obtained indicate that *P. perezi* tadpoles were negatively affected by the exposure to nitrogenous compounds. Nevertheless, the LC_{50} values obtained suggest that larvae of the studied species show some degree of tolerance to the nitrogenous compounds tested. The LC_{50} values obtained for NO_2^- and NO_3^- ions are in all cases higher than the peak concentrations naturally occurring in localities in the Segura River basin where breeding populations of the studied species have been detected (e.g. 74.35 mg NO_2^-/l and 332.74 mg NO_3^-/l, Suárez, pers. com.). This suggests that the present results are not suitable for determining the effect of exposure to these nitrogenous compounds on *P. perezi* in natural settings. Thus, examining the possible sublethal effects of NO_2^- and NO_3^- ions on the studied species would be more ecologically relevant, since sublethal levels of fertilizers have been shown to affect parameters such as time to hatching (de Wijer *et al.*, 2003; Meredith & Whiteman, 2008; Shinn *et al.*, 2008), abnormality rates (Krishnamurthy *et al.*, 2008; Shinn *et al.*, 2008), activity levels (Hecnar, 1995; Xu & Oldham, 1997; Shinn *et al.*, 2008), feeding (Baker & Waights, 1994; Hecnar, 1995; Xu & Oldham, 1997) or habitat use (Huey & Beitinger, 1980; Marco & Blaustein, 1999), which may indirectly affect larval size (de Wijer *et al.*, 2003; Shinn *et al.*, 2008, but see Meredith & Whiteman, 2008) or lead to larval mortality (de Wijer *et al.*, 2003; Shinn *et al.*, 2008, but see Meredith & Whiteman, 2008). Nevertheless, as regards the NH_4^+ ion, the LC_{50} values obtained for each observation period corresponded to lower concentrations than those found in the field in the study area (e.g. 154.6 mg NH_4^+/l, Suárez, pers. com.), which suggests that *P. perezi* populations may be naturally exposed to lethal concentrations of the NH_4^+ ion and, therefore, be potentially suffering a decline as a consequence of eutrophication.

Our LC_{50} estimates confirm the prediction that the risk of dying from a pollutant increases both as the concentration of the toxicant raised (Watt & Jarvis, 1997; Shinn *et al.*, 2008) and with longer exposure times as it has been previously described for different amphibian species (Marco *et al.*, 1999; Schuytema & Nebeker, 1999a, b; Sparling & Harvey, 2006; Shinn *et al.*, 2008). This fact evidences that mortality was not restricted to initial exposure to the different chemicals, but was due to a chronic effect of continuous exposure.

Significant interspecific variation concerning amphibian larvae tolerance to fertilizers has been described (e.g. Hecnar, 1995; Marco *et al.*, 1999). However, most experiments differed greatly in the tested conditions (see Table 3) that largely affect tadpole responses. For instance, larval sensitivity to nitrogenous pollutants varies greatly with exposure time (Marco *et al.*, 1999; Schuytema & Nebeker, 1999a, b; Sparling & Harvey, 2006), developmental stage (Ortiz-Santaliestra *et al.*, 2006), chemical compound employed (Schuytema & Nebeker, 1999a, b) and experimental venue (Egea-Serrano *et al.*, chapters 7 and 9 in the present thesis). Therefore, any reliable comparison between species has to bear in mind such differences in experimental settings. Despite these drawbacks we can affirm that for NO_3^- ion, *P. perezi* can be considered less tolerant than *Xenopus laevis* and *Pseudacris regilla*, but more resistant than other ranids such as *Rana clamitans* or *R. pipiens*. Interestingly, *P. perezi* is generally less tolerant to NH_4^+, but exhibits high resistance to increased concentrations of NO_2^- (Table 3). Inter- and intraspecific physiological studies concerning the effectiveness of detoxification pathways for nitrogenous ions are needed to explain this disparity.

Table 3. LC_{50} values of different chemical forms of ammonium, nitrite and nitrate reported in the literature for larval amphibians. References (in brackets): 1: Marco *et al.* (1999); 2: Hecnar (1995); 3: Xu & Oldham (1997); 4: Schuytema & Nebeker (1999a); 5: Sparling & Harvey (2006); 6: Shinn *et al.* (2008); 7: Present data. *Developmental stages sensu Gosner (1960).

Species	Gosner Stage*/Age	Days of exposure	Nitrogen source	LC_{50}
Ambystoma gracile	Newly hatched	4	$NaNO_2$	6.24 mg NO_2^-/l (1)
	Newly hatched	7	$NaNO_2$	5.06 mg NO_2^-/l (1)
	Newly hatched	15	$NaNO_2$	3.32 mg NO_2^-/l (1)
	Newly hatched	15	KNO_3	103.6 mg NO_3^-/l (1)
Bufo americanus	25	4	NH_4NO_3	60.2-174 mg NO_3^-/l (2)
Bufo boreas	Newly hatched	4	$NaNO_2$	>23.0 mg NO_2^-/l (1)
	Newly hatched	7	$NaNO_2$	17.7 mg NO_2^-/l (1)
	Newly hatched	15	$NaNO_2$	5.75 mg NO_2^-/l (1)
Bufo bufo	32-35	4	NH_4NO_3	1704 mg NO_3^-/l (3)
	32-35	7	NH_4NO_3	1837 mg NO_3^-/l (3)
Bufo calamita	25	15	$NaNO_2$	>24.64 mg NO_2^-/l (6)
Hyla meridionalis	25	5	$NaNO_2$	383.59 mg NO_2^-/l (6)
	25	7	$NaNO_2$	143.20 mg NO_2^-/l (6)
	25	10	$NaNO_2$	65.7<LC_{50}<104.0 mg NO_2^-/l (6)
	25	15	$NaNO_2$	>49.29 mg NO_2^-/l (6)
Hyla regilla	Newly hatched	4	$NaNO_2$	18.07 mg NO_2^-/l (1)
	Newly hatched	7	$NaNO_2$	11.8 mg NO_2^-/l (1)
	Newly hatched	15	$NaNO_2$	4.04 mg NO_2^-/l (1)

Table 3 (continued). LC_{50} values of different chemical forms of ammonium, nitrite and nitrate reported in the literature for larval amphibians. References (in brackets): 1: Marco *et al.* (1999); 2: Hecnar (1995); 3: Xu & Oldham (1999); 4: Schuytema & Nebeker (1999a); 5: Sparling & Harvey (2006); 6: Shinn *et al.* (2008); 7: Present data. *Developmental stage sensu Gosner (1960).

Species	Gosner Stage*/Age	Days of exposure	Nitrogen source	LC_{50}
Pelophylax perezi	14-18	15	$NaNO_2$	$16.4<LC_{50}<49.3$ mg NO_2^-/l (6)
	25	6	$NaNO_2$	419.24 mg NO_2^-/l (6)
	25	7	$NaNO_2$	151.16 mg NO_2^-/l (6)
	25	10	$NaNO_2$	<16.43 mg NO_2^-/l (6)
	18-19	10	$NaNO_2$	48.0 mg NO_2^-/l (6)
	18-19	12	$NaNO_2$	7.15 mg NO_2^-/l (6)
	18-19	16	$NaNO_2$	<1.64 mg NO_2^-/l (6)
	25	4	NH_4Cl	19.27 mg NH_4^+/l (7)
	25	3	$NaNO_2$	914.59 mg NO_2^-/l (7)
	25	3	$NaNO_3$	2381.53 mg NO_3^-/l (7)
Pseudacris regilla	26-27	4	NH_4SO_4	148.24 mg NH_4^+/l (4)
	26-27	10	NH_4SO_4	115.33 mg NH_4^+/l (4)
	26-27	4	NH_4NO_3	599.6 mg NO_3^-/l (4)
	26-27	10	NH_4NO_3	244.5 mg NO_3^-/l (4)
	26-27	4	$NaNO_3$	7749.1 mg NO_3^-/l (4)
	26-27	10	$NaNO_3$	1178.9 mg NO_3^-/l (4)
Pseudacris triseriata	25	4	NH_4NO_3	75.3 mg NO_3^-/l (2)
Rana aurora	Newly hatched	4	$NaNO_2$	18.37 mg NO_2^-/l (1)
	Newly hatched	7	$NaNO_2$	13.14 mg NO_2^-/l (1)
	Newly hatched	15	$NaNO_2$	3.91 mg NO_2^-/l (1)
Rana clamitans	25	4	NH_4NO_3	143.5 mg NO_3^-/l (2)

Table 3 (continued). LC_{50} values of different chemical forms of ammonium, nitrite and nitrate reported in the literature for larval amphibians. References (in brackets): 1: Marco *et al.* (1999); 2: Hecnar (1995); 3: Xu & Oldham (1999); 4: Schuytema & Nebeker (1999a); 5: Sparling & Harvey (2006); 6: Shinn *et al.* (2008); 7: Present data. *Developmental stage sensu Gosner (1960).

Species	Gosner Stage*/Age	Days of exposure	Nitrogen source	LC_{50}
Rana pipiens	25	4	NH_4NO_3	100.1 mg NO_3^-/l (2)
	25	4	NH_4HCO_3	37.1 mg NH_4^+/l (5)
	25	7	NH_4HCO_3	15.6 mg NH_4^+/l (5)
	25	4	NH4ClO4	57.9 mg NH_4^+/l (5)
	25	7	NH4ClO4	29.9 mg NH_4^+/l (5)
Rana pretiosa	Newly hatched	4	$NaNO_2$	22.4 mg NO_2^-/l (1)
	Newly hatched	7	$NaNO_2$	4.27 mg NO_2^-/l (1)
	Newly hatched	15	$NaNO_2$	1.87 mg NO_2^-/l (1)
	Newly hatched	15	KNO_3	72.85 mg NO_3^-/l (1)
Xenopus laevis	26-27	4	NH_4SO_4	173.57 mg NH_4^+/l (4)
	26-27	4	NH_4Cl	163.93 mg NH_4^+/l (4)
	26-27	10	NH_4SO_4	58.5 mg NH_4^+/l (4)
	26-27	10	NH_4Cl	82.19 mg NH_4^+/l (4)
	26-27	4	NH_4NO_3	446 mg NO_3^-/l (4)
	26-27	10	NH_4NO_3	243,3 mg NO_3^-/l (4)
	26-27	4	$NaNO_3$	7332.8 mg NO_3^-/l (4)
	26-27	10	$NaNO_3$	5474.6 mg NO_3^-/l (4)

Finally, the results presented in this study must be considered preliminary for establishing the effects of nitrogenous compounds on *P. perezi* natural populations. Previous studies show the existence of both intraspecific differences (Shinn *et al.*, 2008), and even local genetic adaptation (Johansson *et al.*, 2001; Egea-Serrano *et al.*, 2009) to different chemical water stressors. In addition, ontogenetic differences in sensitivity to fertilizers have been described in amphibians (Ortiz-Santaliestra *et al.*, 2006) and the lethal effects of the exposure to nitrogenous compounds detected in laboratory experiments may be significantly higher than in more natural conditions

(Egea-Serrano *et al.*, chapters 4 and 7 in the present thesis). Furthermore, pollutant exposure could produce sublethal effects on amphibian larvae (Baker & Waigths, 1994; Hatch & Blaustein, 2000; Johansson *et al.*, 2001; de Wijer *et al.*, 2003; Ortiz *et al.*, 2004; Krishnamurthy *et al.*, 2008; Meredith & Whiteman, 2008; Shinn *et al.*, 2008; Egea-Serrano *et al.*, 2009) that could have important implications on population viability (Smith, 1987; Semlitsch *et al.*, 1988). These aspects point to the importance of developing future studies to establish the actual sensitivity of different developmental stages and populations of *P. perezi* to nitrogen compounds.

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CAPÍTULO 4

POPULATIONAL DIVERGENCE IN THE IMPACT OF THREE NITROGENOUS COMPOUNDS AND THEIR COMBINATION ON LARVAE OF THE FROG *PELOPHYLAX PEREZI* (SEOANE, 1885)

Abstract: Pollution by nitrogenous compounds is a putative stressful factor that may be causally linked to the decline of amphibians. One way to understand the potentially detrimental consequences of eutrophication on amphibian populations is to investigate variation among populations differing in exposure to nitrogen, this variation potentially indicating evolutionary potential to cope with this stressor. We have examined the effect of nitrogenous compounds (NH_4^+; NO_2^-; NO_3^-, both alone and in combination) on fitness-related larval traits in four populations of *Pelophylax perezi* naturally exposed to different degrees of eutrophication. The results indicate that both survival and larval final size decrease at higher concentrations of these compounds, either singly or in combination. Additionally, the nitrogenous compounds were more lethal and larval food consumption and final mass were significantly reduced when they were exposed to combinations of compounds. Populations inhabiting highly polluted aquatic environments tolerated higher levels of nitrogenous compounds and showed higher survival rates and larger final size than the populations of less polluted environments, suggesting the potential to adapt to increased nitrogenous contamination in this species.

Key words: Populational divergence, nitrogenous compounds, nitrogenous mixtures, amphibians

INTRODUCTION

An important consequence of human activities is the profound alteration of the global nitrogen cycle, which in many areas increases both the availability and mobility of nitrogen. Fertilizers, animal wastes and atmospheric deposition are the main sources of nitrogenous compounds to the environment worldwide (Vitousek *et al.*, 1997; Ritter & Bergstrom, 2001; Holland *et al.*, 2005; Marco & Ortiz-Santaliestra, in press). As a consequence, pollution by nitrogenous compounds is widespread (Carpenter *et al.*, 1998) and is expected to increase in the future (Tilman *et al.*, 2001; Galloway *et al.*¸ 2003). Thus, assessing the impact nitrogenous pollution on wildlife should be of major concern.

Although amphibian populations may show long-term natural, cyclic demographic fluctuations (Pechmann *et al.*, 1991; Tejedo, 2003), their worldwide decline has been demonstrated (Houlahan *et al.*, 2000; Stuart *et al.*, 2004). Anthropogenic factors such as overexploitation, habitat loss, climatic change and disease have been argued as important causes of amphibian population decline (e.g. Stuart *et al.*, 2004; Beebee & Griffiths, 2005). Among these factors, chemical pollution has been reported a major threat to amphibians (e.g. Beebee & Griffiths, 2005), and there is abundant evidence that these vertebrates are susceptible to the toxic effects of nitrogenous compounds (e.g. Ortiz *et al.*, 2004; Camargo *et al.*, 2005; Marco & Ortiz-Santaliestra, in press).

Previous studies reported that nitrogenous compounds such as ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) caused both lethal and sublethal effects on larval amphibians (e.g. Xu & Oldham, 1997; Marco *et al.*, 1999; De Wijer *et al.*, 2003; Burgett *et al.*, 2007; Griffis-Kyle, 2007). Nitrate is the dominant form of nitrogen in water bodies with aerobic conditions (Camargo *et al.*, 2005), where it is usually accompanied by other stress factors, including other nitrogen forms (e.g. Vidal-Abarca

et al., 2000). Previous studies show that nitrogenous compounds in combination with other environmental factors such as UV-B radiation or pesticides (Hatch & Blaustein, 2000; Boone *et al.*, 2005; Macías *et al.*, 2007) affect amphibian embryos and larvae more severely than when they act alone. Despite the great relevance that such interactions may have on amphibian decline, to date no study on the mixture effects of multiple nitrogenous compounds has been carried out. In the present study, we hypothesized that increasing the number of nitrogenous compounds would negatively affect tadpole survival and performance.

Interspecific variation in nitrogen tolerance appears to be important in amphibian larvae (Marco *et al.*¸1999; Marco & Ortiz-Santaliestra, in press). Nevertheless, information of intraspecific differences among populations in the sensitivity to nitrate compounds is almost totally lacking (but see Hecnar, 1995; Johansson *et al.*, 2001; Hatch & Blaustein, 2003; Shinn *et al.*, 2008). Such information is likely to shed light on the evolutionary potential of nitrogen tolerance and would be therefore crucial for predicting the impact of these compounds on amphibian communities.

In this study we examine the effect of eutrophication on larvae of *Pelophylax perezi*. This species of waterfrog is widespread throughout the Iberian Peninsula and Southern France (Llorente & Arano, 1997) and mainly inhabits permanent water bodies (Díaz-Paniagua, 1990). As a consequence of farming practices (one of the most important nitrogen sources in nature (e.g. Ritter & Bergstrom, 2001), these habitats may hold high concentrations of different nitrogen forms. For instance, in southeastern Iberian Peninsula, concentrations as high as 154.6 mg NH_4^+/l; 74.4 mg NO_2^-/l; 333 mg NO_3^-/l have been recorded (Ballester, 2003; Suárez, personal communication). Because *P. perezi* uses water bodies as shelter from predators (Martín *et al.*, 2006), for foraging (Docampo & Vega, 1990) and as breeding habitat (Egea-Serrano *et al.*¸ 2005), nitrogen

pollution may cause a threat to this species throughout its life cycle. The wide distribution and abundance of *P. perezi* even in highly eutrophic agricultural habitats (Llorente *et al.*, 2002) means that there may be populations exposed to differing nitrogen concentration, which may cause interpopulational variation in tolerance to eutrophication, as has been shown in other species (e.g. Johansson *et al.*, 2001).

The aims of the present study were 1) to determine the effects of exposure to different concentrations of NH_4Cl, $NaNO_2$ and $NaNO_3$, and some of their combinations, on mortality, food consumption and final mass of larvae of *P. perezi*; and, 2) to investigate the divergence in populations exposed to different levels of nitrate pollution that may arise from genetic adaptation in the tolerance to differing levels in nitrogen toxicity.

MATERIAL AND METHODS

Studied populations

Four populations of *P. perezi* located in the Segura River basin (SE Iberian Peninsula) (Fig. 1) were selected for the study. This basin is considered as one of the most arid of the Iberian Peninsula (Vidal-Abarca *et al.*, 1987), and is undergoing an accelerated eutrophication (Ballester, 2003) due to intensive agricultural development in the area during the last decades (Pérez & Lemeunier, 2003). The selected populations were naturally exposed to different levels of nitrogen pollution. Two of these populations, considered control populations (C), were exposed to low nutrient concentrations: Río Chícamo (C1, hereafter) (38º12´N, 001º03´W; 170.3 m.a.s.l.), a permanent headwater stream with less than 5.1 mg $N\text{-}NO_3^-$/l (Vidal-Abarca *et al.*, 2000); Rambla Tejera (C2, hereafter) (38º11´N, 002º07´W; 1197.9 m.a.s.l.), a semipermanent headwater stream

with less than 2.1 mg NO_3^-/l (A. Egea-Serrano, unpublished data). The environment of these populations corresponds to bush on marls (C1) or to a mixture of bush and pine trees on limestone lithology (C2). As a contrast to the above populations, two polluted populations (P) were selected: Rambla del Garruchal (37º57´N, 001º04´W; 346.0 m.a.s.l.) (herafter P1), a semipermanent headwater stream which has been exposed to nitrate concentrations as high as 162.1 mg NO_3^-/l for 22 years (Ballester, 2003) due to intensive farming activities and subsequent run-off in its basin, and Campus of Espinardo (38º01´N, 001º10´W; 96.3 m.a.s.l.) (P2, hereafter), a small artificial pool located at the Espinardo Campus of the University of Murcia. Although no data concerning nutrient concentration levels are available for this population, episodic blooms of filamentous algae occurring at least for 10 years (unpublished data) suggest substantial levels of eutrophication. The natural environment of P1 corresponds to pine trees on heterogeneous carbonated materials although most of the stream course has been largely modified and is dominated by intensive livestock farming. The P2 population environment corresponds to a suburban landscape. The geographical separation between populations ranged from 12.0 to 95.2 km.

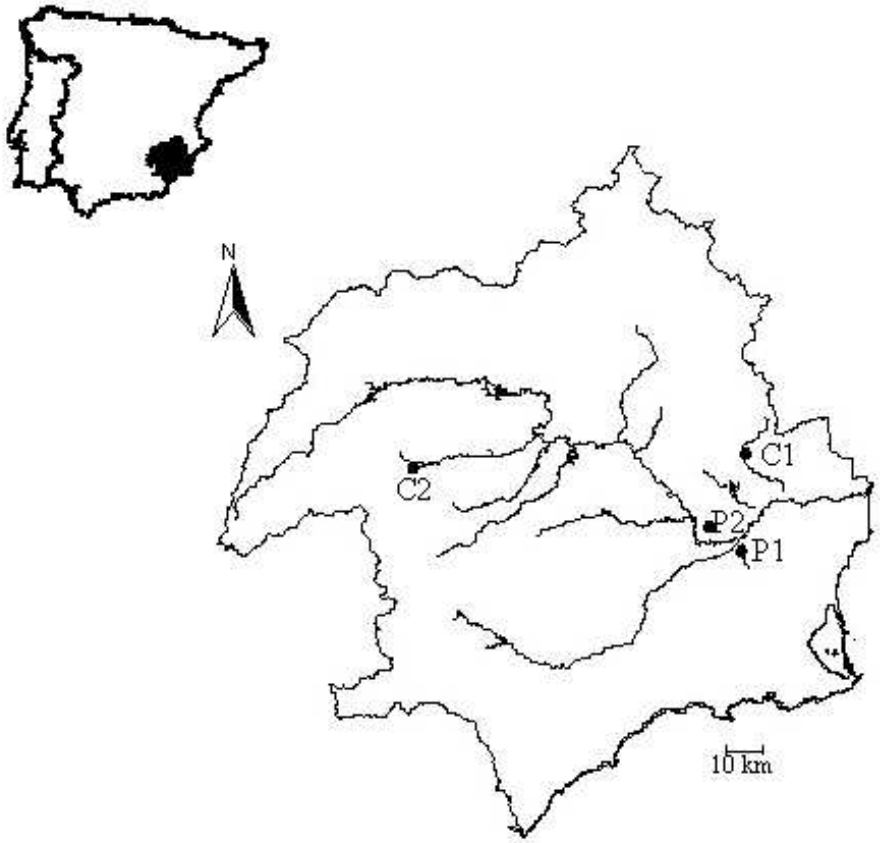

Fig. 1. Location of the studied populations in the Segura River basin. Main water courses in the basin are represented. Control populations: C1 (Río Chícamo) and C2 (Rambla Tejera); polluted populations: P1 (Rambla del Garruchal) and P2 (Campus of Espinardo, University of Murcia).

Experimental design and response variables

Five different egg masses of *P. perezi* were collected from each of the studied populations during April 4-7th 2006. The developmental stage of embryos collected ranged from stage 15 to 20 (Gosner, 1960), with no differences among populations (Chi-square, $P > 0.05$). In all cases, embryos were transported before hatching to the laboratory, where they were reared in 12 l glass aquaria containing dechlorinated tap water (pH = 8.39; conductivity = 985 $\mu S\ cm^{-1}$; 0.002 mg NO_2^-/l; 4.69 mg NO_3^-/l). Embryos from the same population of origin were pooled and reared in the same aquarium. Fully randomly selected larvae at Gosner stage 25 were individually transferred to 1 L plastic beakers containing 500 ml of the treatment solutions shown in Table 1. Each beaker was fully randomly assigned to one out of eight shelves in the laboratory. For the four studied populations the exposure started at the same time. Larvae were reared individually during the experiment. A sample of 13-15 experimental beakers were randomly chosen for each treatment to measure water pH, temperature (ºC), conductivity ($\mu S\ cm^{-1}$) and salinity ($g\ L^{-1}$). Measurements were taken just after applying treatments for first time. Ammonium, nitritre and nitrate concentrations were prepared using NH_4Cl, $NaNO_2$ and $NaNO_3$, respectively, and dechlorinated tap water. For each ion, two concentrations (low or high, Table 1) were selected to assess and magnify any subtle effect that may not have been detected at lower concentration. These concentrations are ecologically relevant, since they were representative of peak concentrations naturally occurring in the field in the Segura River basin (e.g. point sample: 154.6 mg NH_4^+/l; 74.4 mg NO_2^-/l; 333 mg NO_3^-/l, Suárez, personal communication). Since a major objective of the present study was to assess the impact of the treatments on sublethal parameters (food consumption and final mass), the concentrations selected for ammonium and nitrite were lower than those cited for the

field because preliminary tests showed that higher concentrations produced high larval mortality after short-term exposure (Egea-Serrano *et al.*, 2009). Larvae were fed every three days with dry dog chow pellets (250-350 mg).

Table 1. Nitrogen treatments used in this study.

Treatment	Concentration
1	0
2[*1a]	1.35 mg NH_4^+/l
3[*2a]	13.5 mg NH_4^+/l
4[**1a]	6.67 mg NO_2^-/l
5[**2a]	66.7 mg NO_2^-/l
6[***1a]	36.47 mg NO_3^-/l
7[***2a]	364.7 mg NO_3^-/l
8[*1b; **1b; ***2b]	1.35 mg NH_4^+/l + 364.7 mg NO_3^-/l + 6.67 mg NO_2^-/l
9[*1b; **2b; ***2b]	1.35 mg NH_4^+/l + 364.7 mg NO_3^-/l + 66.7 mg NO_2^-/l
10[*2b; **1b; ***2b]	13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 6.67 mg NO_2^-/l
11[*2b; **2b; ***2b]	13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 66.7 mg NO_2^-/l

*Treatments selected for the analysis of the separate effect of ammonium: 1: low concentration; 2: high concentration; a: ammonium isolated; b: ammonium combined with other nitrogenous compounds.

** Treatments selected for the analysis of the separate effect of nitrite: 1: low concentration; 2: high concentration; a: nitrite isolated; b: nitrite combined with other nitrogenous compounds.

*** Treatments selected for the analysis of the separate effect of nitrate: 1: low concentration; 2: high concentration; a: nitrate isolated; b: nitrate combined with other nitrogenous compounds.

Larvae were exposed to the chemicals for 21 consecutive days in a laboratory at a roughly constant temperature (25ºC) and with indoor lighting of 12:12h dark:light cycle. Water was renewed and the treatments restored every two days. The position of the beakers on the shelves was fully randomly re-assigned after each renewal. For C1 and C2 populations, each treatment was replicated seven times (i.e. seven larvae per treatment), whereas for P1 and P2 populations we had five replicates (i.e. five larvae per treatment) due to the limited number of larvae. Larval mortality at the end of the experiment was recorded and surviving larvae were weighed with an electronic balance (±0.0001 g) after being blotted dry. Additionally, at days 7, 14 and 21, an estimate of larval food consumption was taken using the methodology proposed by Rist *et al.* (1997). Thus, a preweighed dried dog chow pellet (250-350 mg) was placed in each beaker with no other food source available. After 24 h, the uneaten food was removed, dried again for 24 h at 50 ºC and weighed. To correct for any bias in the estimates due to the loss of food by its handling or physical dilution in water a correction factor was calculated. To do so, a pilot study was performed with 50 dried dog chow pellets (250-350 mg). Each one was weighed, left in water without larvae for 24 h and weighed again after being dried for 24h h at 50 ºC. Final dried mass of the chow pellets (y) was regressed against their initial dried mass before being submerged (x), obtaining the following regression equation: $y = -0.0082+0.8725x$ ($R^2 = 0.988$, $P = 0.0001$, $N=50$). The initial dried mass of each chow pellet (x) was introduced in this regression model to determine its final dried mass after removing the loss due to handling or dilution (y). The difference between this mass and the final dried mass weighed after chow pellets were in the experimental beakers with the larvae provided the amount of food eaten by each larva.

Analysis of data

The physicochemical characteristics of the water (dependent variables) were analysed separately using one-way ANOVAs with treatment as factor. Larval mortality was analysed by Generalized Linear Models (GLZ) for binary data fitting a binomial distribution of the data with a Logit Link function to yield maximum-likelihood ratio estimates, using environment of origin (non-polluted, involving C1 and C2 populations, vs polluted, involving P1 and P2 populations), population (nested within environment), treatment and their interaction as factors. Because GLZ analyses do not allow random factors, the mentioned factors were all considered as fixed factors. Since individuals were pooled and then fully randomly assigned to treatments, the random factor family could not be included in the analysis. Food consumption, measured as the mean value of the absolute amount of food eaten by each surviving larva for the three estimates taken, and final larval mass were analysed separately by General Linear Models (GLM), using the fixed factors treatment and environment of origin, and the random factor population (nested within environment), as well as their interactions, as independent factors. We used post hoc HDS Tukey tests for pair-wise comparisons for the treatment factor in the case of the response variables food consumption and larval final mass. Additionally, the effect of compound concentration and mixture on the response variables was analysed in more detail for each nitrogenous compound separately using GLZ (in the case of larval mortality) and GLM (for food consumption and final mass). To do so, treatments containing the compound of interest were selected and assigned to the proper category of the factors concentration (low or high) and mixture (single or combined with the rest of compounds, regardless their concentration) (Table 1). Environment of origin, concentration, mixture and their different interactions were included as fixed factors. To analyze for the effect of population of origin, this random factor was also included in

the analyses, nested within environment (in the case of larval mortality it was considered as a fixed factor, as mentioned above). Significance levels were not corrected for multiple comparisons because comparisons among different pollutants were based on a priori hypotheses.

All variables were log-transformed (log(x + 1)) except mortality. GLZ for binary data were performed using STATISTICA 6.0 statistical package (Statsoft, Inc. 2001). The rest of the analyses were performed with SPSS® v. 15.0 statistical package. In all cases a significant level of 5% was selected and descriptive statisticals were expressed as mean ± 1 SE.

RESULTS

Water physicochemical characteristics

Water pH and temperature ranged from 6.85 to 8.04 and from 20.0 to 24.8 ºC, respectively. Nitrogen treatments did not affect these parameters ($P > 0.10$ in both cases). Water conductivity ($F_{10,149} = 390$; $P = 0.0001$) and salinity ($F_{10,149} = 524.33$; $P = 0.0001$) differed across treatments, being significantly greater at high concentrations of ammonium, nitrite and nitrate, whether isolated or combined (Table 2).

Table 2. Physicochemical characteristics (mean ± 1 SE) of the water used in the nitrogen treatments. Lower case letters indicate homogenous groups in pairwise comparisons (HDS Tukey´s test, α= 5%). Only variables significantly affected by treatments are shown.

Treatment	Conductivity (μS cm^{-1})	Salinity (gr l^{-1})
1 (n=15)	1265.73±4.85a	0.41±0.007a
2 (n=15)	1274.80±4.79a	0.41±0.007a
3 (n=15)	1345.80±4.93b	0.50±0b
4 (n=15)	1288.33±7.54a,b	0.42±0.011a
5 (n=15)	1394.13±9.82b	0.50±0b
6 (n=14)	1321.21±5.85a,b	0.48±0.011b
7 (n=15)	1799.93±43.58c	0.78±0.011c
8 (n=15)	1883.07±10.95e	0.80±0c
9 (n=13)	1994.15±10.16d,f	0.88±0.012d,e
10 (n=13)	1966.08±8.93e,f	0.84±0.014c,e
11 (n=15)	2076.00±11.08d	0.90±0d

Effects on mortality

Larval mortality was significantly affected by nitrogen treatment and environment of origin (Table 3). Treatments involving high concentrations of ammonium, both isolated and combined with other nitrogenous compounds (treatments 3, 10 and 11), increased larval mortality in relation to the rest of treatments (Fig. 2). This severe effect of high concentration of ammonium compared with lower concentration was also observed when the effects of this compound were analysed separately (Table 4). In addition, the separate analysis on the treatments with nitrite showed that exposure to nitrite in combination with ammonium and nitrate significantly increased larval mortality as

compared to exposure to nitrite only (Table 4, Fig. 2). Larvae from non-polluted populations showed higher mortality than polluted ones (Fig. 2), as revealed both by the analysis of all treatments (Table 3) and by the analyses performed for each nitrogenous compound separately (Table 4). The significant environment x treatment interaction (Table 3) revealed that the pattern of divergence in larval mortality between different environments of origin differed across treatments. Polluted populations had lower larval mortalities than reference populations only for high levels of ammonium, whether isolated or in combination (Fig. 2, treatments 3, 10 and 11, Table 4, significant environment x concentration interaction) suggesting differential sensitivity to ammonium between source environments, possibly indicating local adaptation to this toxicant. This environment-specific response also arose when the effect of ammonium was analysed separately, which also revealed that the exposure to the combination of nitrogenous compounds aggravated the effects of isolated ammonium to some degree, as suggested by the significant concentration x mixture interaction (Table 4).

Table 3. Summary statistics for the GLZ and GLM analyses on mortality, food consumption and final mass of larvae of *P. perezi* in the laboratory. Significant values are shown in bold.

Variable	Source of variation	df	X^2	P	
Mortality	Environment	**1**	**15,481**	**0.0001**	
	Population(Environment)	2	3,223	0.200	
	Treatment	**10**	**65,667**	**0.0001**	
	Environment x Treatment	**10**	**23,068**	**0.011**	
	Population(Environment) x Treatment	20	21,887	0.347	
	Source of variation	df numerator	df denominator	F	P
Food consumption	Environment	1	1.991	12.319	0.073
	Population(Environment)	2	16.866	0.573	0.575
	Treatment	**10**	**17.987**	**121.553**	**0.0001**
	Environment x Treatment	**10**	**18.058**	**5.949**	**0.001**
	Population(Environment) x Treatment	**17**	**166**	**3.048**	**0.0001**
Final weight	Environment	1	2.000	1.829	0.309
	Population(Environment)	**2**	**24.454**	**13.020**	**0.0001**
	Treatment	10	20.382	1.721	0.144
	Environment x Treatment	10	20.453	0.881	0.565
	Population(Environment) x Treatment	19	169	1.445	0.112

Table 4. Summary statistics for the GLZ and GLM analyses on the response variables studied for particular nitrogenous elements: NH_4^+, NO_2^-, and NO_3^-. Significant values are shown in bold. ndf: numerator degrees of freedom; ddf: denominator degrees of freedom.

				NH_4^+				NO_2^-				NO_3^-	
Variable	Source of variation	ndf		X^2	P	ndf		X^2	P	ndf		X^2	P
MORTALITY	Environment: ENV	1		**20.109**	**0.0001**	1		**11.644**	**0.0006**	1		**11.206**	**0.0008**
	Population(ENV)	2		4.697	0.0955	2		**7.877**	**0.0195**	2		2.193	0.3340
	Concentration: CON	1		**27.866**	**0.0001**	1		0.044	0.8331	1		1.140	0.2856[a]
	Mixture: MIX	1		0.222	0.6377	1		**8.997**	**0.0027**	1		3.031	0.0817[b]
	CON x MIX	1		**4.059**	**0.0439**	1		0.001	0.9813	NM		NM	NM
	ENV x CON	1		**7.926**	**0.0049**	1		3.059	0.0802	1		1.786	0.1814[a]
	ENV x MIX	1		0.111	0.7391	1		0.506	0.4767	1		0.895	0.3340[b]
	ENV x CON x MIX	1		1.497	0.2210	1		0.546	0.4598	NM		NM	NM
	Source of variation	ndf	ddf	F	P	ndf	ddf	F	P	ndf	ddf	F	P
FOOD CONSUMPTION	Environment: ENV	1	2.933	0.412	0.5676	1	2.155	**19.032**	**0.0425**	1	32.597	**11.948**	**0.0015**
	Population(ENV)	2	85	1.618	0.2043	2	96	1.066	0.3485	2	77	0.088	0.9157
	Concentration (CON)	1	85	**33.135**	**0.0001**	1	96	**5.768**	**0.0182**	1	36	**105.685**	**0.0001**[a]
	Mixture (MIX)	1	85	**1289.463**	**0.0001**	1	96	**1176.560**	**0.0001**	1	77	**63.787**	**0.0001**[b]
	CON x MIX	1	85	**63.139**	**0.0001**	1	96	**89.873**	**0.0001**	NM		NM	NM
	ENV x CON	1	85	0.449	0.5048	1	96	**49.508**	**0.0001**	1	36	0.087	0.7696[a]
	ENV x MIX	1	85	**4.746**	**0.0321**	1	96	0.537	0.4654	1	77	0.242	0.6239[b]
	ENV x CON x MIX	1	85	1.746	0.1898	1	96	**70.063**	**0.0001**	NM		NM	NM
MASS	Environment: ENV	1	2,117	2.785	0.2302	1	2,031	4.034	0.1804	1	2,167	1.141	0.3897
	Population(ENV)	2	91	**11.430**	**0.0001**	2	101	**7.430**	**0.0009**	2	83	**9.669**	**0.0002**
	Concentration (CON)	1	91	**7.122**	**0.0090**	1	101	**5.082**	**0.0263**	1	36	0.224	0.6386[a]
	Mixture (MIX)	1	91	0.027	0.8699	1	101	0.002	0.9634	1	83	1.724	0.1928[b]
	CON x MIX	1	91	0.856	0.3573	1	101	**6.512**	**0.0122**	NM		NM	NM
	ENV x CON	1	91	0.006	0.9372	1	101	0.433	0.5118	1	36	1.337	0.2548[a]
	ENV x MIX	1	91	1.530	0.2192	1	101	0.501	0.4807	1	83	0.127	0.7225[b]
	ENV x CON x MIX	1	91	0.151	0.6988	1	101	1.027	0.3132	NM		NM	NM

[a] These sources of variation were estimated only for isolated NO_3^-; [b] These sources of variation were estimated only for high concentration of NO_3^-; NM: non measurable sources of variation.

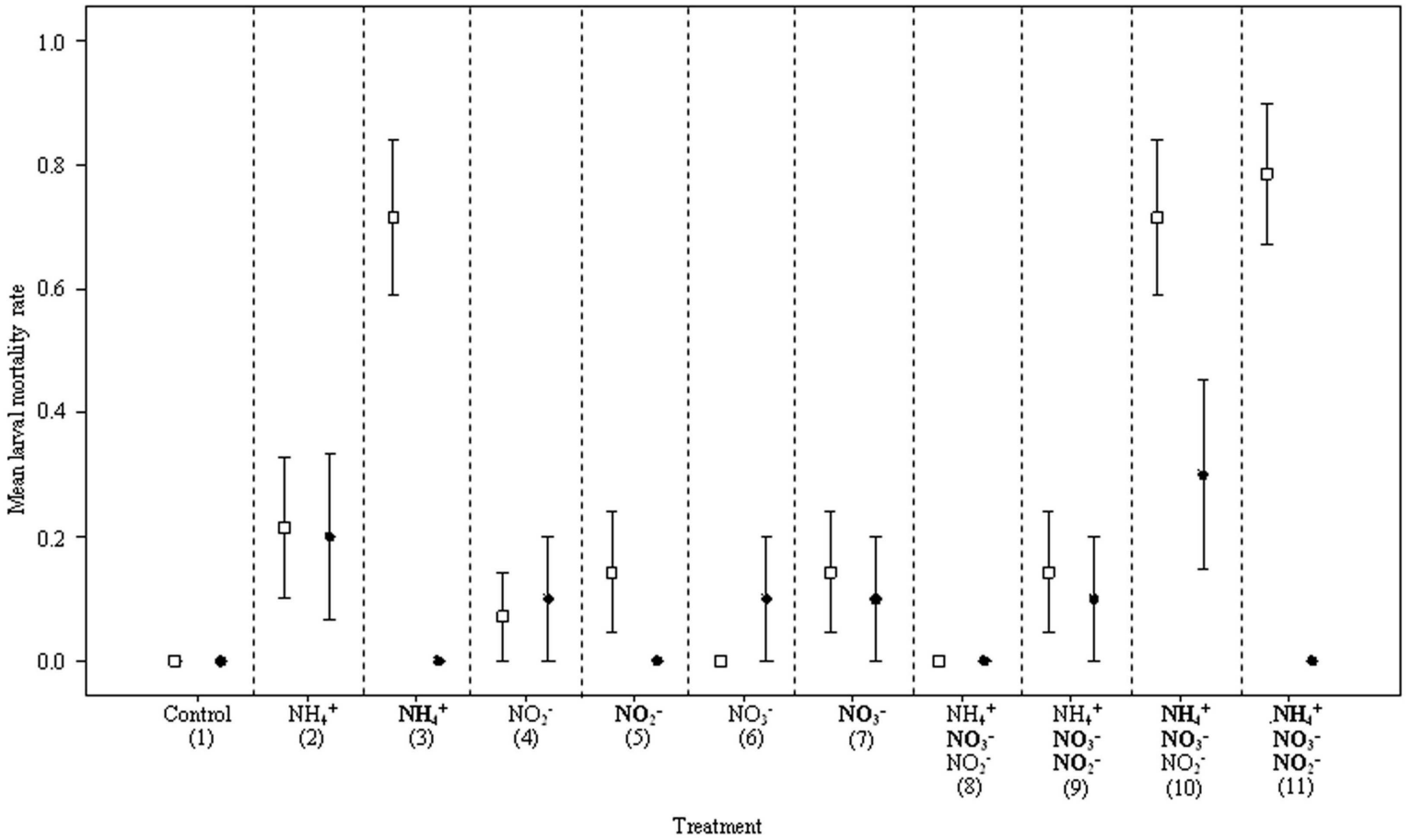

Fig. 2. Larval mortality rate (mean ± 1 SE) for larvae of *P. perezi* from control (□) and polluted (●) populations exposed to low and high concentrations of three nitrogenous compounds and to different combinations thereof. Text in bold indicates the highest concentration for the different ions used in the present study. Treatment codes are shown in brackets (see Table 1 for details).

Effects on averaged food consumption

The amount of food consumed by larvae was significantly affected by nitrogen treatment (Table 3). Exposure to nitrogenous compounds produced lower feeding rates than in control larvae (planned comparison, $F_{1,166} = 292.024$, $P = 0.0001$). Additionally, larvae exposed to nitrogenous mixtures consumed significantly less food than those exposed to individual pollutants (Fig. 3), as revealed by the analysis of the effect of all treatments (Table 3) and by the separate analysis of the effect of each nitrogenous compound (Table 4). Food consumption responses differed among the studied populations (significant environment x treatment interaction, $P < 0.001$, Table 3). Polluted populations ate more food than non-polluted populations in the treatments with higher levels of nitrite (Fig. 3, treatments 5 and 11), whereas the amount of food consumed was lower than in the case of non-polluted populations for the treatment involving low concentration of ammonium (Fig. 3, treatment 2). When separate analyses were performed for each nitrogenous compound, larvae from polluted populations ate more food than non-polluted populations when they were exposed to ammonium in combination with nitrite and nitrate (Table 4, Fig. 3, treatments 8-11) and when they were exposed to high levels of nitrite (Table 4, Fig. 3, treatments 5, 9 and 11). Moreover, significant concentration x mixture interactions were observed for the separate analysis for ammonium and nitrite (Table 4), suggesting that the combination of nitrogenous compounds severely reduced the amount of food consumed by larvae as compared to the exposure to ammonium and nitrite alone.

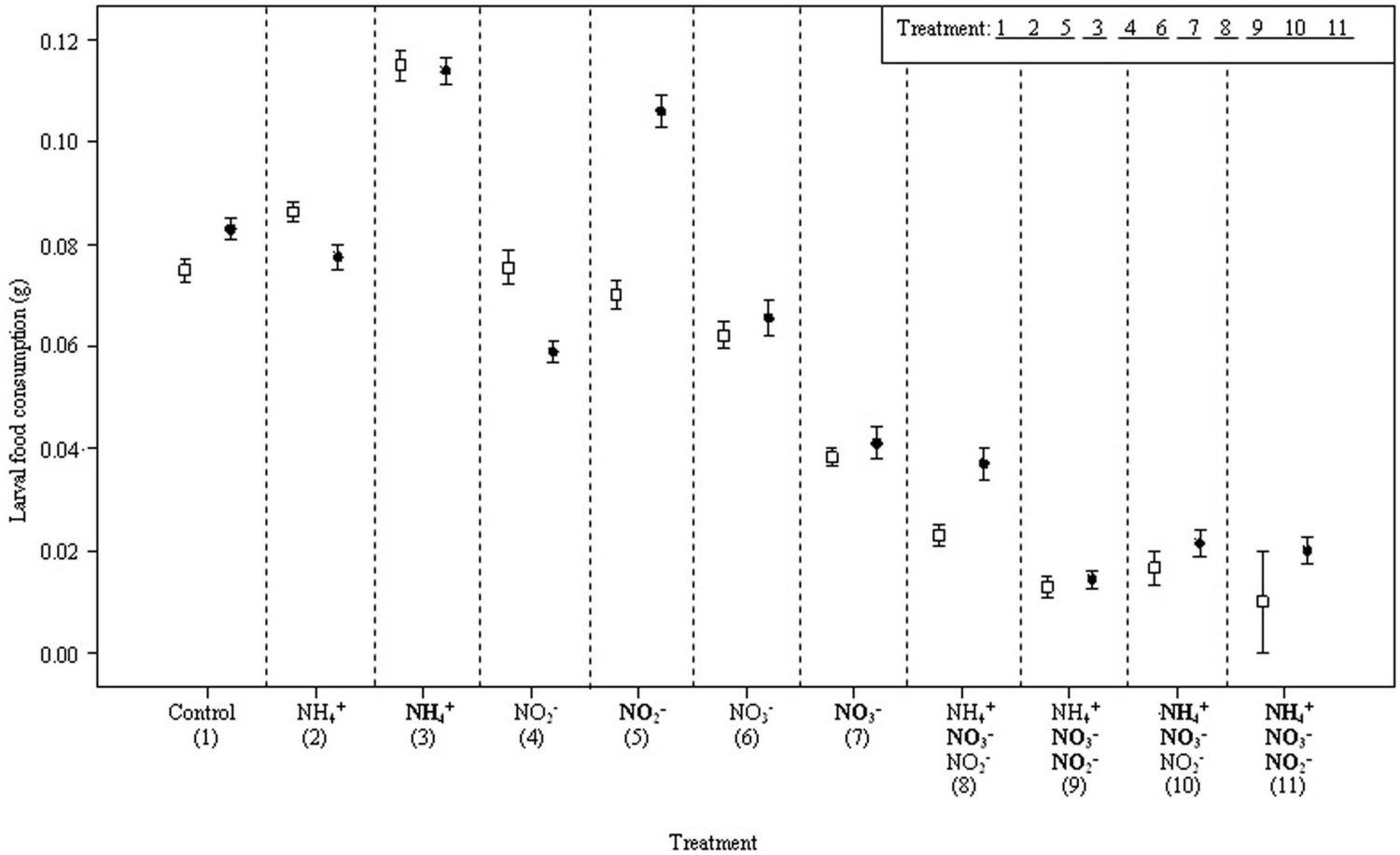

Fig. 3. Mean (± 1 SE) amount of food consumed by larvae of *P. perezi* from control (□) and polluted (●) populations exposed to low and high concentrations of three nitrogenous compounds and to different combinations thereof. Text in bold indicates the highest concentration for the different ions used in the present study. Treatment codes are shown in brackets (see Table 1 for details). Common underbar notation indicates the results of HDS Tukey´s test (α= 5%) performed for the factor treatment (treatment codes underlined by the same bar correspond to homogenous groups).

Effects on final mass

Final larval mass was unaffected by treatment, environment of origin, or their interaction. Only population (nested within environment) significantly affected final mass (Table 3). Larvae from P1 reached a greater size (final mass, mean ± 1SE: 0.350 ± 0.023 g, N = 53) than larvae from the other populations (final mass, mean ± 1SE: C1: 0.178 ± 0.017 g, N = 55; C2: 0.191 ± 0.013 g, N = 57; P2: 0.211 ± 0.019 g, N = 47) (Table 3). This pattern was also observed when the effect of ammonium, nitrite and nitrate was analysed separately (Table 4). These separate analyses, however, revealed that the treatments with high concentrations of ammonium and nitrite led to lower final mass in the tadpoles than lower concentrations of these toxicants (Table 4, Fig. 4). Moreover, this decrease in mass at high concentration of nitrite was more pronounced when nitrite was combined with other nitrogen forms (Table 4, Fig. 4, treatments 9 and 11).

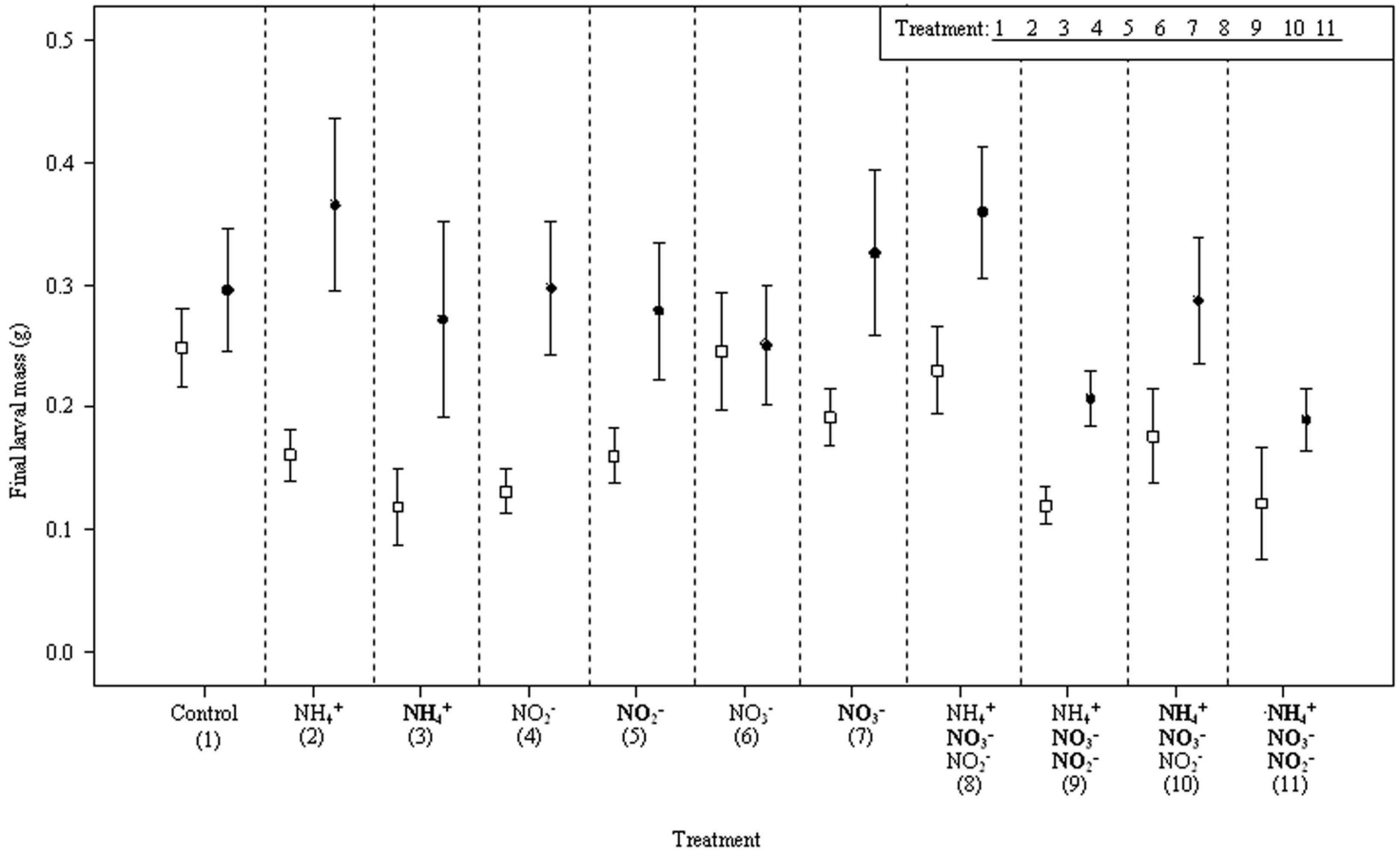

Fig. 4. Mean final larval mass (± 1 SE) for larvae of *P. perezi* from control (□) and polluted (●) populations exposed to low and high concentrations of three nitrogenous compounds and to different combinations thereof. Text in bold indicates the highest concentration for the different ions used in the present study. Treatment codes are shown in brackets (see Table 1 for details). Common underbar notation indicates the results of HDS Tukey´s test (α= 5%) performed for the factor treatment (treatment codes underlined by the same bar correspond to homogenous groups).

DISCUSSION

The results obtained indicated three basic conclusions: (1) Exposure of *P. perezi* tadpoles to nitrogenous treatments strongly reduced larval survival and food consumption and caused a slight decrease in final larval mass. (2) Exposure to pollutant combinations produced more severe effects in relation to nitrogenous compounds acting isolately for the response variables larval mortality and food uptake. (3) A noticeable degree of interpopulational variation was detected for larval mortality and food consumption; larvae from polluted sites being more tolerant than those from low nitrate stress environments.

Previous studies have reported that exposure to high levels of ammonium (Jofre & Karasov, 1999; Schuytema & Nebeker, 1999a), nitrite (Marco *et al.*, 1999; Griffis-Kyle, 2005, 2007; Macías *et al.*, 2007; Shinn *et al.*, 2008) and nitrate (Baker & Waights, 1993, 1994; Schuytema & Nebeker, 1999a,b; Smith *et al.*, 2005) increase larval amphibian mortality. The present study indicates that high concentrations of ammonium, whether isolated or combined with other nitrogen compounds, caused significant mortality. Moreover, when the effects of each compound were analysed separately, a significant increase of larval mortality was detected when nitrite acted in combination with ammonium and nitrate as compared to its effect as a single stressor. This apparent lack of effect of nitrate and nitrite when they act as single stressors supports previous studies on several frog species (Smith *et al.*, 2004;Vaala *et al.*, 2004; Smith, 2007). The existence of an effective detoxification pathway may explain the observed tolerance to nitrite and nitrate ions, as has been hypothesized for *Lithobates catesbeianus* tadpoles suffering short-term nitrite exposure (Huey & Beitinger, 1980a). Additionally, the chlorides present in the dechlorinated tap water used in the experiment

(110 mg Cl^-/l) (or any other monovalent ion) may have competed with nitrite for ionic uptake sites on the respiratory surface (Huey & Beitinger, 1980a,b), reducing its overall uptake and, consequently, its effects. These considerations emphasize the importance of performing physiological studies to identify the mechanisms underlying the divergent tolerance levels of the studied species to different nitrogenous compounds.

The general reduction in food ingestion observed in the presence of high nitrogenous concentrations also supports previous studies (Baker & Waights, 1993, 1994; Hecnar, 1995; Xu & Oldham, 1997; but see Watt & Oldham, 1995). Since treatments were renewed every two days, preventing algal growth, no alternative food source was available in the experimental beakers. Moreover, the effects observed cannot be attributed to the fact that larvae exposed to polluted treatments were more inactive, since larval activity level increased for those treatments significantly affecting this response variable (Egea-Serrano *et al.*, chapter 5 in the present thesis). Thus, the observed lower food consumption may either be regarded as a disturbance response of the symbiotic gut bacteria involved in digestion (Hecnar, 1995) or an alteration in the chemosensory system of the larvae, which is essential for food detection (Veeranagoudar *et al.*, 2004). However, these arguments do not explain the contrary trend observed in larvae exposed to high concentrations of ammonium and nitrite (for polluted populations), which increased their food uptake. A possible explanation for this enhanced ingestion may be the need to satisfy the increased energetic costs that detoxification pathways would demand (Wright & Wright, 1996).

The exposure to nitrogenous compounds has been shown to produce a diversity of responses in amphibian larvae growth in laboratory conditions. Some studies observed no significant effect of fertilizers on final larval size (Hecnar, 1995; Vaala *et al.*, 2004; Smith *et al.*, 2005), whereas others mentioned that such exposure reduced the final size

of the larvae (e.g. Griffis-Kyle, 2007; Shinn *et al.*, 2008). Considering the separate analyses for each compound, our results would support this last scenario (at least in the case of ammonium and nitrite), although no significant effects were detected when the data for the eleven treatments included in the present study were considered jointly. As treatments significantly affected the amount of food consumed, we predicted that treatments where larvae consumed greater amounts of food would lead to a larger final mass. However, this prediction was not supported by any significant relationship between final mass and food consumed (Pearson correlation: $r = 0.042$, $P = 0.546$, $n = 206$). Larval growth may be affected by the physicochemical characteristics of water such as pH or temperature (Álvarez & Nicieza, 2002; Glos *et al.*, 2003). In our study, these parameters did not significantly differ and, as a consequence, their effect on the results recorded may be insignificant. Nevertheless, those treatments corresponding to high concentration of nitrate, as well as to the mixtures of nitrogenous compounds, showed significantly higher water conductivity and salinity than the rest. Exposure to these conditions may have induced osmotic stress that affected growth, such as described in previous studies (Gómez-Mestre *et al.*, 2004; Ortiz-Santaliestra, 2008) and which may have biased the results obtained. In addition, the increased energetic costs that detoxification pathways may involve in the case of the exposure to high concentrations of ammonium and, for P1 and P2 populations, nitrite (Wright & Wright, 1996) would probably have led to a lack of effect on final mass, in spite of the increased amount of food consumed by larvae exposed to these treatments. Further physiological studies are needed to identify the mechanisms responsible for the apparent lack of effect of individual pollution treatments on final larval mass in *P. perezi*. Nevertheless, to fully assess the consequences of nitrogenous pollution on larval mass in the studied species, experiments in more natural environments are needed since nitrogenous

compounds may have indirect effects through the alteration of primary producers and microbial communities (Carpenter *et al.*, 1998; Fenn *et al.*, 2003), which may produce positive effects on the mass of surviving tadpoles (e.g. De Wijer *et al.*, 2003).

The exposure to cocktails of fertilizers and other stressing factors, such as pesticides (Boone *et al.*¸ 2005), UV-B radiation (Hatch & Blaustein, 2000, 2003; Macías *et al.*, 2007) or low pH (Hatch & Blaustein, 2000) may affect amphibian larvae more severely than when they are exposed to these factors acting in isolation (but see Boone & Bridges-Britton, 2006). The response to a cocktail of stressors may be either additive or synergistic (Berenbaum, 1989). In the present study, the exposure of larvae to the combination of different nitrogenous compounds at different concentrations affected larval mortality and food consumption more severely than the exposure to single compounds, in contrast to previous studies in which no different effects were recorded in relation to those produced by isolated factors (Orton *et al.*, 2006) or control treatments (Boone & Bridges-Britton, 2006). Moreover, the separate analyses performed for each one of the nitrogenous compounds studied suggests that the exposure to the combination of such compounds would have a synergistic effect on mortality, food consumption and even final mass. Nevertheless this interpretation needs to be considered cautiously because the greater effect of the combination of nitrogenous compounds may be due to their higher total amount of nitrogen, rather than to a true interaction among compounds. More research involving pairwise combinations of pollutants is needed to evaluate the effects of nitrogenous mixtures independently of the cumulative nitrogen effects and to properly assess whether such mixtures interact synergisticaly or additively.

The response to nitrogenous pollution differed between populations that were naturally exposed to different levels of nitrogenous pollution in their aquatic habitats.

As regards larval mortality, two populations breeding in polluted habitats expressed higher tolerance to ammonium both alone and in combination with nitrate and nitrite than two other reference populations exposed to lower nutrient concentrations in their environments. This result would suggest that populations breeding in habitats exposed to high levels of toxicant nitrogenous compounds may have evolved rapidly in response to environmental nitrification in a pronounced process of selection. This hypothetical rapid evolution, presumably caused by the intense farming activities developed in the polluted areas during the past three decades, is compatible with the idea that environmental stress, especially stress of contemporary anthropogenic origin, such as chemical pollution, is a strong force generating local adaptations and rapid evolution (Hoffmann & Hercus, 2000; Meyer & Di Giulio, 2003; Carroll *et al.*, 2007). Previous studies revealed interpopulational variations in the tolerance to fertilizers of different amphibian species (Hecnar, 1995; Johansson *et al.*, 2001; Hatch & Blaustein, 2003; Macías *et al.*, 2007; Shinn *et al.*, 2008), and local adaptation has also been suggested (e.g. Johansson *et al*., 2001, in *Rana temporaria* populations). In our case, the studied populations have been exposed to different nutrient concentration only during the last few decades (Vidal-Abarca *et al.*, 2000; Ballester, 2003). Additionally, they belong to the same river basin and are located relatively close to each other, which may preclude strong demographic isolation between them. Although we have no information on population genetic structure, we could hint that the pattern of population divergence in *P. perezi* populations in nitrogenous compounds tolerance is concordant with an adaptive process involving natural selection. However, some caution is needed because the differences among populations do not appear consistent with regard to their origin, since the pattern observed for larval mortality does not ensue in the case of food consumption and final mass. Moreover, other factors may mask the divergent responses.

For instance, egg masses were collected directly in the field, and, therefore, we can not discount the possibility that selective mortality and/or acclimatization process could be responsible for the responses observed (Freda & Dunson, 1984; Räsänen *et al.*, 2003). Furthermore, no data exist on the heritable genetic mechanisms for nitrogenous compound tolerance and so, population divergence may be the response to other environmentally based sources such as maternal effects (Räsänen & Kruuk 2007). All these considerations make it difficult to assess the interpretation of an adaptive response of populations to increased levels of eutrophication.

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CAPÍTULO 5

EXAMINING THE IMPACT OF THREE NITROGENOUS COMPOUNDS AND THEIR COMBINATION ON INACTIVITY LEVEL AND HABITAT USE OF LARVAE OF *PELOPHYLAX PEREZI* (SEOANE, 1885)

Abstract: Several studies have assessed the effects of nitrogenous compounds on amphibian behavior. However, few have focused on the effects of their combination with other stressing factors or on the variation of the tolerance to pollutants among populations. We analyzed the effect of nitrogenous compounds (NH_4^+; NO_2^-; NO_3^-, both alone and in combination) on larval behavior (inactivity level and habitat use) in four populations of *Pelophylax perezi* naturally exposed to different levels of eutrophication. The number of censuses larvae were found inactive or on the bottom of the experimental beakers decreased at lower concentrations of these compounds acting singly or when low concentration of NO_2^- was combined with NH_4^+ and NO_3^-. Additionally, the combination of nitrogenous compounds affected more severely the response variables than when they acted singly according to an additive model. Populations inhabiting highly polluted aquatic habitats marginally showed lower inactivity level than the populations of less polluted environments, which would suggest the potential to adapt to differing levels of nitrogenous compounds. However, such conclusion regarding the studied variables is not conclusive since environment-specific response was not recorded for habitat use, in spite of the intra-specific variation observed for this response variable.

Key words: Populational divergence, nitrogenous compounds, nitrogenous mixtures, amphibians, habita use, inactivity

INTRODUCTION

Human activities, such as farming practices or fossil fuel combustion, may increase the concentration of nitrogenous compounds in the environment (Vitousek *et al*., 1997; Ritter & Bergstrom, 2001). As a consequence, pollution by such compounds is widespread (Carpenter *et al.*, 1998) and is expected to increase in the future (Tilman *et al.*, 2001; Galloway *et al.*¸ 2003). This consideration emphasizes the relevance of performing studies to accurately assess the effects of nitrogenous pollutants on amphibian populations, since such pollutants have been suggested as a major cause for amphibian decline in several regions of the world (Berger, 1989; Hamer *et al.*, 2004).

The number of studies devoted to analyse the impact of nitrogenous compounds is growing (see revision Marco & Ortiz-Santaliestra, in press). As a result, both lethal and sublethal effects on amphibian larvae have been reported for ammonium, nitrite and nitrate (e.g. Xu & Oldham, 1997; Marco *et al.*, 1999; Griffis-Kyle, 2007). Nevertheless, the great inter- (Marco *et al.* 1999) and intraspecific variation (Johansson *et al.*, 2001, Egea-Serrano *et al.*, 2009) described in relation to the tolerance to nitrogenous compound exposure makes of great relevance to develop further studies to complete the existing database (Smith *et al.*, 2005), which would allow to perform proper management strategies to warrant the conservation of this group of vertebrates.

In spite of most ecotoxicological studies deal with the effects of an isolated pollutant (Storfer, 2003), the exposure to the combination of different stressing factors may exacerbate the impact of such factors acting isolately through additive or synergistic responses (Berenbaum, 1989). As regards to fertilizers, their combination with others stressing factors, such as pesticides (Boone *et al.*¸ 2005), UV-B radiation (Hatch & Blaustein, 2000, 2003; Macías *et al.*, 2007) or low pH (Hatch & Blaustein,

2000) may affect amphibian larvae more severely than when they are exposed to these factors acting isolated. In the field, nitrogenous compounds are present in combination with complex cocktails of other factors, including other nitrogenous compounds (e.g. Vidal-Abarca *et al.,* 2000). Nevertheless, the number of studies analysing the impact that different combinations of nitrogenous compounds may have on amphibian larvae is very scarce (Egea-Serrano *et al.*, 2009).

Behavior may be defined as the physical manifestation of an organism´s physiological response to its environment (Clotfelter *et al.*, 2004). Larval developmental stages of amphibians have been showed to reduce their activity as a consequence of the exposure to stressing factors, such as pesticides (Bridges, 1997, 1999), UV-B radiation (Hatch & Blaustein, 2000) or fertilizers (Hecnar, 1995; Xu & Oldham, 1997; Hatch & Blaustein, 2000; Shinn *et al.*, 2008). This effect may represent severe adverse consequences for individuals, since a decrease in tadpole activity may lead to reduced feeding rates (i.e. reduced energy intake) (Horat & Semlitsch, 1994) and competitive ability (Dayton & Fitzgerald, 2001), affecting so growth and development (Alford & Harris, 1988). This can make larvae vulnerable to pond dessication (Bridges, 1997) or to predators by lenghtening larval period (Wilbur *et al.*, 1983). Apart from activity, pollutants such as nitrogenous compounds may affect habitat use by larvae, which may also influence feeding rate (Warkentin, 1992) (and its implications, previously described) of larvae and their risk of being consumed by a predator (Tarr & Babbitt, 2002). The impact that these considerations may represent on long-term population viability makes of great importance to include behavioral endpoints in ecotoxicological studies (Hatch & Blaustein, 2000), specially considering that they are easy to record and that they may be more sensitive than other parameters (Warner *et al.*, 1966).

The aims of the present study were 1) to determine the effects of the exposure to different concentrations of ammonium chloride, sodium nitrite and sodium nitrate, and to some of their combinations on inactivity and habitat use of larvae of *Pelophylax perezi*; and, 2) to evaluate whether there are evidences of divergence in populations undergoing different levels in nitrogenous pollution that may ground for genetic adaptation in the tolerance to differing levels in nitrogen toxicity.

P. perezi is a waterfrog that widespread through the Iberian Peninsula and Southern France (Llorente & Arano, 1997) inhabits mainly permanent water bodies (Díaz-Paniagua, 1990). These habitats, as a consequence of farming practices (one of the most important nitrogen sources in the nature (e.g. Ritter & Bergstrom, 2001), may hold high concentrations of different nitrogen forms (e.g. for southeastern Iberian Peninsula, point simple sample: 154.6 mg NH_4^+/l; 74.4 mg NO_2^-/l; 333 mg NO_3^-/l, Suárez, personal communication). So, this species might be threatened by nitrogen pollution through all its life cycle, because it uses water bodies as shelter from predators (Martín *et al.*, 2006) and as foraging (Docampo & Vega, 1990) and breeding habitat (Egea-Serrano *et al.*¸ 2005). Nevertheless, the wide distribution range of *P. perezi* makes possible the existence of interpopulational variation as regards its tolerance to different levels of eutrophication, as has been shown in other settings (Johansson *et al.*, 2001; Egea-Serrano *et al.*, 2009). This consideration, together with the existence of different nitrogenous ions in the field which may act jointly, points out the relevance of analysing the interpopulational variation and the effect of multiple nitrogen forms on behavioral endpoints to accurately assess the impact of nitrogenous pollution on a widespread distributed amphibian species.

MATERIAL AND METHODS

Studied populations

Five different egg masses of *P. perezi* were collected from four populations located in the Segura River Basin in the first fortnight of April 2006. This basin has been reported as one of the most arid of Iberian Peninsula (Vidal-Abarca *et al.*, 1987), and a trend towards eutrophication of the water bodies located in it has been described (Ballester, 2003). The selected populations were naturally exposed to highly different levels of nitrogen pollution. Two of these populations, corresponding to the permanent headwater stream Río Chícamo (38º12´N, 001º03´W; 170.3 m.a.s.l.) and to the semipermanent headwater stream Rambla Tejera (38º11´N, 002º07´W; 1197.9 m.a.s.l.) (C1 and C2 hereafter), showed low nutrient concentration (C1: less than 5.1 mg N-NO_3^-/l (Vidal-Abarca *et al.*, 2000); C2: less than 2.1 mg NO_3^-/l, unpublished data). The terrestrial environment of these populations corresponds to bush on marls (C1) or to a mixture of bush and pine trees on limestone lithology (C2). In contrast to the previous populations, the third one is located in another semipermanent headwater stream, Rambla del Garruchal (37º57´N, 001º04´W; 346.0 m.a.s.l.) (herafter P1), which has been exposed at least for the last 22 years to nitrate concentration as high as 162.1 mg NO_3^-/l (Ballester, 2003) due to intensive farming activities and subsequent run-off in its basin. In addition, its terrestrial environment corresponds with pine trees on heterogeneous carbonated materials but through most of the course of the stream the habitat has been largely modified, being dominated by intensive cattle exploitations. The fourth population corresponds to a small artificial pool located at the Campus of Espinardo of University of Murcia (38º01´N, 001º10´W; 96.3 m.a.s.l.) (P2) and no data concerning nutrient concentration levels are available. Nevertheless, episodic blooms of filamentous algae

suggest important levels of eutrophication. Since this population is located at the campus, its environment corresponds to a periurban landscape. The geographical separation between populations ranged from 12.0 km to 95.2 km.

Experimental design and response variables

Developmental stage of embryos when they were collected ranged from 15 to 21 Gosner´s stage (Gosner, 1960), not existing differences among populations in developmental stage (Chi-square, $P > 0.05$). Embryos were reared in 12 l glass aquaria containing dechlorinated tap water (pH= 8.39; conductivity= 985 µS/cm; 0.002 mg NO_2^-/l; 4.69 mg NO_3^-/l). When they reached Gosner´s 25 developmental stage, they were individually transferred to 1 l plastic beakers containing 500 ml of the treatment solutions showed in Table 1. A sample of 13-15 experimental beakers were randomly selected for each treatment to measure water pH, temperature (ºC), conductivity (µS/cm) and salinity (g/l). Measurements were taken just after restoring treatments for first time. Ammonium, nitritre and nitrate concentrations were prepared using NH_4Cl, $NaNO_2$ and $NaNO_3$, respectively, and dechlorinated tap water. For each ion two concentrations (low or high) were selected to assess and magnify any subtle effect that may have not been detected at lower concentration. In all cases, these concentrations were representative of those naturally occurring in the field in the Segura River basin (e.g. point simple sample: 154.6 mg NH_4^+/l; 74.4 mg NO_2^-/l; 333 mg NO_3^-/l, Suárez, personal communication). Larvae were fed every three days with dry dog chow pellets (250-350 mg).

Table 1. Treatments to larvae of *Pelophylax perezi* were exposed in the present study.

Treatment	Concentration
1	0
2	1.35 mg NH_4^+/l
3	13.5 mg NH_4^+/l
4	6.67 mg NO_2^-/l
5	66.7 mg NO_2^-/l
6	36.47 mg NO_3^-/l
7	364.7 mg NO_3^-/l
8	1.35 mg NH_4^+/l + 364.7 mg NO_3^-/l + 6.67 mg NO_2^-/l
9	1.35 mg NH_4^+/l + 364.7 mg NO_3^-/l + 66.7 mg NO_2^-/l
10	13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 6.67 mg NO_2^-/l
11	13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 66.7 mg NO_2^-/l

Larvae were exposed to the chemicals for 21 consecutive days in a laboratory at a roughly constant temperature (25º C) and with indoor lighting of 12:12 h dark:light cycle. Water was renewed and treatments restored every two days. For C1 and C2 populations, each treatment was replicated seven times, whereas for the rest of studied populations, was five times due to differences in embryo survival. At days 2, 4, 7, 14 and 21 after the beginning of the experiment, inactivity (defined as absence of tail or feeding movements) and habitat use (recorded whether larvae were on the bottom of the experimental beakers) were recorded by point simple method.

Water physicochemical characteristics were analysed separately using one-way ANOVA with treatment as factor. Larval inactivity and habitat use were analysed separately by General Linear Models (GLM). Number of censuses larvae were detected either inactive or on the bottom of the experimental beakers were used as dependent variables and environment of origin (control vs polluted), population (nested within environment), treatment and their interactions as independent factors. Only larvae surviving at the end of the experiment were included in the analyses. We used post hoc HSD Tukey tests for pair-wise comparisons for the treatment factor. Additionally, each nitrogenous compound was analysed separately for each studied variable to examine in more detail the effects of compound concentrations and mixtures. To do so, treatments containing the compound of interest were selected and assigned the proper category of the factors concentration (low or high) and mixture (single or combined with the rest of compounds, regardless their concentration). Environment of origin, concentration, mixture and their different interactions were included as factors. To analyze for the effect of population of origin, this factor was also included in the analyses, nested within environment.

All variables were log- transformed (log(x + 1)). Analyses were performed with SPSS® v. 15.0 statistical package. In all cases a significant level of 5% was selected and descriptive statisticals were expressed as mean ± 1 SE.

RESULTS

Water physicochemical characteristics

Treatments did not affect either water pH ($F_{10,149}$= 1.4; P= 0.167) or temperature ($F_{10,149}$= 0.4; P= 0.925). Nevertheless, water conductivity ($F_{10,149}$= 390; P= 0.0001) and

salinity ($F_{10,149}$= 524.33; P= 0.0001) were significantly higher for those treatments showing high concentrations of ammonium, nitrite and nitrate, isolated or combined (Table 2).

Table 2. Measured water physicochemical characteristics (mean ± 1SE) for the treatments used in the present study. Lowercase letters indicate homogenous groups of pairwise comparisons (HDS Tukey´s test, α= 5%) for the effect of treatment on the physicochemical characteristics of water in the experimental beakers.

Treatment	Conductivity (μS/cm)	Salinity (gr/l)
1 (n=15)	1265.73±4.85^{a}	0.41±0.007^{a}
2 (n=15)	1274.80±4.79^{a}	0.41±0.007^{a}
3 (n=15)	1345.80±4.93^{b}	0.50±0^{b}
4 (n=15)	1288.33±7.54^{a,b}	0.42±0.011^{a}
5 (n=15)	1394.13±9.82^{b}	0.50±0^{b}
6 (n=14)	1321.21±5.85^{a,b}	0.48±0.011^{b}
7 (n=15)	1799.93±43.58^{c}	0.78±0.011^{c}
8 (n=15)	1883.07±10.95^{e}	0.80±0^{c}
9 (n=13)	1994.15±10.16^{d,f}	0.88±0.012^{d,e}
10 (n=13)	1966.08±8.93^{e,f}	0.84±0.014^{c,e}
11 (n=15)	2076.00±11.08^{d}	0.90±0^{d}

Effects on inactivity

Treatments significantly affected larval inactivity (Table 3). The number of censuses that larvae exposed to control treatment were recorded inactive was higher than for those exposed to treatments 6, 8 and 10. Additionally, the exposure to treatment 8 reduced larval inactivity in relation to treatment 5 (Fig. 1). Although population (nested within environment) did not affected larval inactivity, the environment of origin showed

marginal effects ($F_{1,2.006}$= 15.531; P= 0.059; Table 3), being larvae from control environments detected inactive more number of censuses. The results obtained does not suggest the existence of population-specific tolerance to the treatments considered, since neither the interaction Environment x Treatment nor Treatment x Population(Environment) were significant (Table 3).

Table 3. Summary statistics for the GLM analyses performed on habitat use and inactivity level of larvae of *Pelophylax perezi* exposed to different nitrogenous treatments (significant values appear in bold).

Source of Variation	df numerator	df denominator	F	P
Inactivity				
Environment	1	2.006	15.531	0.059
Population (Environment)	2	26.880	1.405	0.263
Treatment	**10**	**21.162**	**4.124**	**0.003**
Environment x Treatment	10	21.264	1.089	0.413
Treatment x Population (Environment)	19	171	1.037	0.422
Habitat use				
Environment	1	1.999	0.152	0.734
Population (Environment)	2	23.505	2.143	0.140
Treatment	10	20.262	2.069	0.079
Environment x Treatment	10	20.320	0.169	0.997
Treatment x Population (Environment)	**19**	**171**	**1.760**	**0.031**

Separate analyses for each compound revealed that treatment enriched with high concentrations of ammonium (treatments 3, 10, 11) or nitrite (treatments 5, 9, 11) led to higher larval inactivity level than lower concentrations of these ions (Table 4, Fig. 1). Moreover, the combination of nitrite with other nitrogenous compounds (treatments 8-11) reduced the inactivity level of larvae in relation to its effect acting isolately (Table 4, Fig. 1). This response was more pronounced for polluted populations than for control populations (Table 4; Fig. 1).

Table 4. Summary statistics for the GLM analyses on inactivity and habitat use of larvae of *P. perezi* in the laboratory for particular nitrogenous elements: NH_4^+, NO_2^-, and NO_3^- , individually and in combination. Significant values are shown in bold. ndf:numerator degrees of freedom; ddf: denominator degrees of freedom.

		NH_4^+				NO_2^-				NO_3^-			
Variable	Source of variation	ndf	ddf	F	P	ndf	ddf	F	P	ndf	ddf	F	P
Inactivity	Environment: ENV	1	3.220	**9.6485**	**0.0482**	1	2.250	11.9281	0.0629	1	3.089	8.8621	0.0566
	Population(ENV)	2	92	1.2739	0.2846	2	103	0.9886	0.3756	2	105	1.2391	0.2939
	Concentration (CON)	1	92	**3.9837**	**0.0489**	1	103	**8.9837**	**0.0034**	1	105	3.5761	0.0614[a]
	Mixture (MIX)	1	92	0.8252	0.3661	1	103	**9.0825**	**0.0032**	1	105	3.5391	0.0627
	CON x MIX	1	92	0.0704	0.7913	1	103	0.4912	0.4850	NM	NM	NM	NM
	ENV x CON	1	92	1.0735	0.3029	1	103	0.1120	0.7386	1	105	0.7125	0.4005[a]
	ENV x MIX	1	92	0.8058	0.3717	1	103	**4.8799**	**0.0294**	1	105	1.5885	0.2103
	ENV x CON x MIX	1	92	0.4612	0.4988	1	103	0.0006	0.9806	NM	NM	NM	NM
Habitat use	Environment: ENV	1	2.479	0.4248	0.5699	1	2.189	0.0212	0.8965	1	2.177	0.5167	0.5415
	Population(ENV)	2	92	3.0316	0.0531	2	103	1.3019	0.2764	2	105	**6.9447**	**0.0015**
	Concentration (CON)	1	92	1.5575	0.2152	1	103	2.3766	0.1262	1	105	**5.7715**	**0.0180[a]**
	Mixture (MIX)	1	92	**9.7227**	**0.0024**	1	103	1.4023	0.2391	1	105	0.2478	0.6197
	CON x MIX	1	92	0.0026	0.9594	1	103	0.0567	0.8123	NM	NM	NM	NM
	ENV x CON	1	92	0.0135	0.9079	1	103	0.0764	0.7828	1	105	0.0001	0.9924[a]
	ENV x MIX	1	92	0.0653	0.7989	1	103	1.1588	0.2842	1	105	0.1965	0.6585
	ENV x CON x MIX	1	92	0.3223	0.5716	1	103	0.1275	0.7218	NM	NM	NM	NM

[a] These sources of variation were estimated only for high concentration of NO_3^-; NM: non measurable sources of variation.

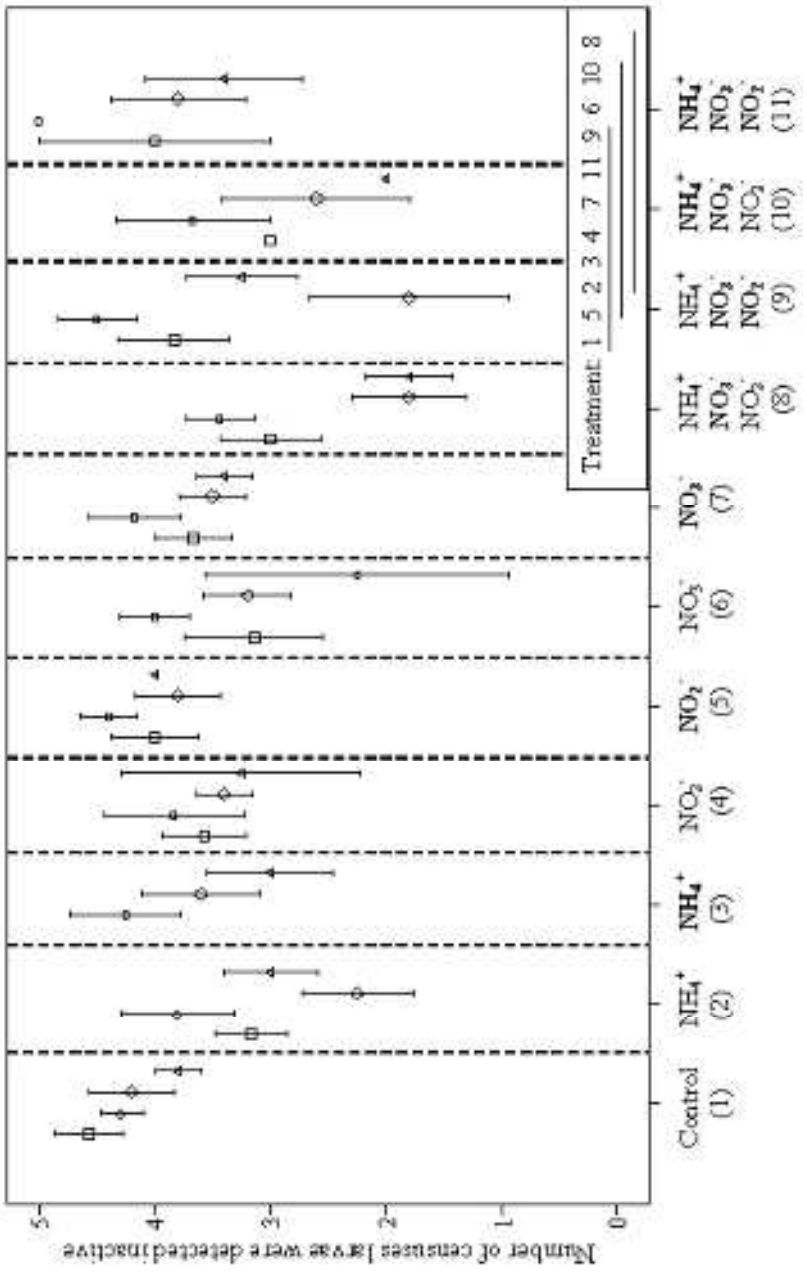

Fig. 1. Mean larval inactivity (± 1 SE) for four different *Pelophylax perezi* populations exposed to low and high concentrations of three nitrogenous compounds and to different combinations of them. Text in boldface indicates the higher concentration for the different ions used in the present study. Treatment codes are shown in brackets. Results of HDS Tukey´s test (α = 5%) performed for the factor treatment are shown (common underbars notation). □C1; ○C2; ◇P1; △P2.

Effects on habitat use

Treatment marginally affected larvae habitat use (Table 3), being larvae exposed to control treatment detected on the bottom of the experimental beakers higher number of censuses than those exposed to treatments 2, 4, 6, 8 (Fig. 2) and, marginally (HSD Tukey test: P= 0.063), to treatment 3. Neither environment of origin nor population significantly affected larvae habitat use. Although the Environment x Treatment interaction was not significant, a Population-specific tolerance x Treatment was found (Table 3; Fig. 2). Additionally, larvae exposed to the combination of nitrogenous compounds (treatments 8-11) increased the number of censuses they were found on the bottom of the beakers in relation to the exposure to ammonium acting isolately (Table 4; Fig. 2).

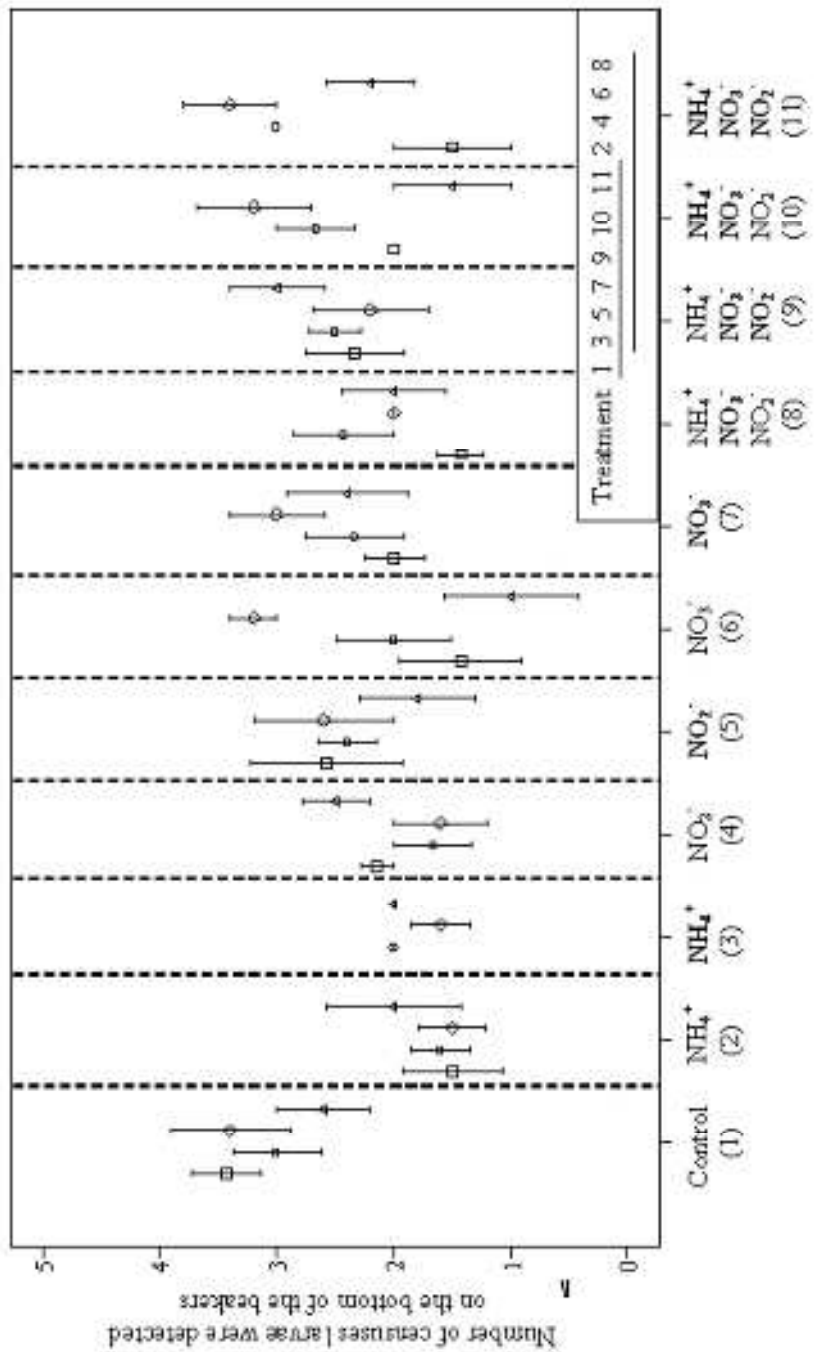

Fig. 2. Mean larvae habitat use (± 1 SE) for four different *Pelophylax perezi* populations exposed to low and high concentrations of three nitrogenous compounds and to different combinations of them. Text in boldface indicates the higher concentration for the different ions used in the present study. Treatment codes are shown in brackets. Results of HDS Tukey´s test (α = 5%) performed for the factor treatment are shown (common underbars notation). □C1; ○C2; ◇P1; △P2.

DISCUSSION

The exposure to low concentration of nitrate, acting alone, or to the combination of low concentration of nitrite with nitrate and ammonium reduced the number of censuses that larvae of *P. perezi* were detected inactive. Moreover, the number of times larvae were detected on the bottom of the experimental beakers was reduced by the exposure to low concentration of ammonium or nitrate, acting alone, or to low concentrations of nitrite, both alone and combined with nitrate and ammonium. These results may be explained by considering the oxydation of hemoglobin to methemoglobin, a response described for larval *Rana catesbeiana* exposed to nitrite (Huey & Beitinger, 1980a). Methemoglobin cannot join oxygen, which lead to low concentration of arterial oxygen (Jensen, 2003). As described for anuran species (i.e. *Rana catesbeiana*, *Xenopus laevis*), the development in hypoxic environments makes larvae to increase the frequency of atmospheric oxygen uptake (Wasserssug & Feder, 1983; Feder & Wasserssug, 1984; Crowder *et al.*, 1998), response also described for larval amphibians when exposed to nitrite (Huey & Beitinger, 1980b; Marco & Blaustein, 1999). So, an hypothetical increase of methemoglobin concentration could contribute to explain the fewer number of censuses that larvae exposed to polluted treatments were detected on the bottom of the beakers. Moreover, atmospheric oxygen uptake may represent positive buoyancy (Wasserssug & Feder, 1983; Feder & Wasserssug, 1984), which would contribute to keep larvae far from the bottom. These aspects (atmospheric oxygen demand and derived positive buoyancy) would also explain the detected decrease in larval inactivity in response to the exposure to treatments, as the positive correlation between both behavioral variables suggests (Pearson correlation: $r= 0.391$, $P= 0.0001$, $n = 214$). Thus, larvae would need to increase both tail undulation frequency to compensate positive

buoyancy (Van Bergeijk, 1959) and swimming performance through water column to access to its surface to uptake atmospheric oxygen. The response found in our study contrast with the decrease of larval activity reported in previous publications (Hecnar, 1995; Xu & Oldham, 1997; Hatch & Blaustein, 2000; Shinn *et al.*, 2008). Such disagreement may be due to the fact that the treatments selected in such studies may have produced more severe effects on the experimental individuals than those used in the present study. Thereby, exposed larvae would have not been able to increase their performance to satisfy the physiological costs of the exposure to the treatments. This consideration may contribute also to explain the lack of significant differences between larvae exposed to the highest polluted treatments when compared with control larvae. Anyway, the effects of the treatments on larval *P. perezi* may represent important consequences for indidual survival and fitness since larvae of this species feed mainly on the bottom of the water bodies, at least in laboratory conditions (Díaz-Paniagua, 1987). This fact suggests that nitrogenous pollutants may increase the risk of being consumed by a predator and reduce the food intake, as described by Egea-Serrano *et al.* (2009), which can also indirectly affect developmental rate and larval period duration (Alford & Harris, 1988).

The combination of nitrogenous compounds produced more severe effects on larval habitat use or inactivity than when ammonium or nitrite acted isolated, respectively. This scenario supports previous studies describing more severe effects when nitrogenous pollutants were combined with other stressing factors such as pesticides, UV-B radiation or low pH (Hatch & Blaustein, 2000, 2003; Boone *et al.*¸ 2005; Macías *et al.*, 2007; but see Boone & Bridges-Britton, 2006; Orton *et al.*, 2006). Since no significant concentration x mixture interactions were detected, the effects observed would correspond to an additive model (Berenbaum, 1989), in contrast to

other study with *P. perezi* (Egea-Serrano *et al.*, 2009) in which synergistic effects on mortality, food consumption and mass were found.

The divergence in populational reaction norms in larval habitat use suggests the existence of geographical variation in relation to the tolerance of larvae of *P. perezi* to nitrogenous compounds, in accordance to previous studies (Hecnar, 1995; Johansson *et al.*, 2001; Hatch & Blaustein, 2003; Macías *et al.*, 2007; Shinn *et al.*, 2008; Egea-Serrano *et al.*, 2009). Since the studied populations may have been exposed to different nutrient concentration (Vidal-Abarca *et al.*, 2000; Ballester, 2003), an adaptive process, and even genetic adaptation, could be expected, as it has been previously described for *Rana temporaria* (Johansson *et al.*, 2001) and even for *P. perezi* larvae (Egea-Serrano *et al.*, 2009). However, the existence of different responses to treatments between control and polluted populations was only detected for larval inactivity, and exclusively when nitrite was analysed separately. Thus, the general lack of responses to the treatments differing for the studied variables between control and polluted environments does not support the local adaptation hypothesis. However, Egea-Serrano *et al.* (2009) described the existence of an adaptive process when studying larval mortality and food consumption for *P. perezi*. This disagreement could be attributed to a higher sensitivity to pollution of the studied behavioral endpoints in relation to other lethal and sublethal parameters, which may have masked the existence of any environment-specific tolerance to nitrogenous compounds.

Finally, it has to be noticed that the concentrations of ammonium, nitrite and nitrate used in the present study, although high, represent levels naturally occurring in the field in the Segura River Basin (154.6 mg NH_4^+/l; 74.4 mg NO_2^-/l; 333 mg NO_3^-/l, Suárez, personal communication). This fact suggests that *P. perezi*, although it has been described as tolerant to pollution (Llorente *et al.*, 2002), may be threatened by

eutrophication. Nevertheless, it is difficult to extrapolate results obtained in laboratory conditions to the field (Ortiz *et al.*, 2004). Moreover, although the experiment design would correspond to a long-term exposure (sensu Marco & Ortiz-Santaliestra, in press), the effects of longer exposures cannot be inferred. Additionally, the effects of pollution on amphibians depends on many factors such as the compound responsible for such pollution (Schuytema & Nebeker, 1999a,b), amphibian developmental stage (Griffis-Kyle, 2005; Ortiz-Santaliestra *et al.*¸ 2006), the presence of other stressing factors (Hatch & Blaustein, 2000; Boone *et al.*, 2005; Macías *et al.*, 2007) and the impact of pollutants on food sources (Watt & Oldham, 1995; Boone *et al.*, 2005). These considerations emphasize the urgence of performing future research to assess the actual impact of nitrogenous pollution on larvae of *P. perezi* in more realistic designs.

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CAPÍTULO 6

ANALYSIS OF THE AVOIDANCE OF NITROGEN FERTILIZERS IN THE WATER COLUMN BY JUVENILE IBERIAN WATER FROG, *PELOPHYLAX PEREZI* (SEOANE, 1885), IN LABORATORY CONDITIONS

Abstract: In an experiment carried out in the laboratory in beakers, the avoidance of ammonium chloride, isolated or combined with sodium nitrite and sodium nitrate, in aquatic habitat by froglets of *Pelophylax perezi* was studied. The results obtained suggest that nitrogen polluted treatments were not avoided by froglets of the studied species. However, despite the non- avoidance of the aquatic environment as a consequence of the presence of nitrogen compounds, significant inter-individual variation in treatment avoidance was detected. Although these results are not conclusive, they would suggest that froglets of *P. perezi* may occupy habitats which contain high levels of organic compounds and that they differ in their level of avoidance to fertilizer exposure.

Key words: Nitrogenous compounds, treatment avoidance, *Pelophylax perezi*, postmetamorphic individuals

INTRODUCTION

Fertilizer pollution has been described as one of the major factors threatening amphibian populations throughout the world (Stuart *et al.*, 2004). Both aquatic and terrestrial amphibian phases are vulnerable to nitrogen excess in their environments (Hatch *et al.*, 2001; Marco *et al.*, 2001; Ortiz *et al.*, 2004; Griffis-Kyle, 2007). Ammonium chloride and sodium nitrate are nitrogen compounds used as fertilizer (Bhandari *et al.*, 1971; Graebing *et al.*, 2002) since they are a source of ammonium and nitrate. The presence of these ions in water may increase nitrite concentration as a consequence of bacterial activity (Atlas & Bartha, 2002). All three forms of nitrogen have been shown to negatively (Jofre & Karasov, 1999; Schuytema & Nebeker, 1999; Griffis-Kyle, 2007) or positively, by way of algal proliferation (Boone *et al.*, 2007) affect the survival, growth and development of amphibian embryos and larvae. Nevertheless, no study has been performed on the effects of their excess on postmetamorphic amphibians in spite of the great importance that postmetamorphic stages may have on amphibian population dynamics, as it has been previously suggested (Biek *et al.*, 2002). Previous studies have shown that amphibians with an adult terrestrial phase can detect fertilizers in their environment and avoid them (Hatch *et al.*, 2001; Marco *et al.*, 2001; Ortiz-Santaliestra *et al.*, 2005). However, as regards species that use aquatic or semiaquatic habitats during their postmetamorphic stages as shelter from predators (Martín *et al.*, 2006), for foraging (Docampo & Vega, 1990) and as breeding habitat (Egea-Serrano *et al.*, 2005), the possible avoidance of such habitats as a response to fertilizer exposure has not been assessed.

Moreover, in natural conditions fertilizers combine with other stressing factors (UV-B radiation, nitrogen compounds, pesticides) and so exposure to such a cocktail

may modify the response in a non-additive way (Brown & Spence, 2003). However, to date most studies have examined the effects of individual contaminants on amphibians (Storfer, 2003) and, although some studies have assessed the effects of a combination of fertilizers and other pollutants (Brown & Spence, 2003; Orton *et al.*, 2006), none has attempted to determine the effects nitrogen compound mixtures have on amphibian behavior.

The aim of the present study was to determine whether the presence of a high concentration of ammonium chloride, isolated or combined with sodium nitrite and sodium nitrate, in the water column was avoided by postmetamorphic *Pelophylax perezi*. This anuran is an endemic waterfrog species from the Iberian Peninsula and Southern France (Llorente & Arano, 1997). It mainly inhabits permanent waterbodies (Diaz-Panigua, 1990), from which adults disperse less than five meters, although juveniles and subadults may move greater distances (Lizana *et al.*, 1989). Because such environments, as a result of farming practices (one of the main nitrogen sources in the environment, e.g. Ritter & Bergstrom, 2001), may hold high concentrations of nitrogenous compounds (e.g. for southeastern Iberian Peninsula: 154.6 mg NH_4^+/l, 74.4 mg NO_2^-/l, 333 mg NO_3^-/l, Suárez, personal communication) which may affect mortality and the behavior of larval *P. perezi* (Egea-Serrano *et al.*, 2009, chapters 4 and 5 in the present thesis), this species might be threatened by organic pollution, since it uses waterbodies as shelter from predators (Martín *et al.*, 2006), for foraging (Docampo & Vega, 1990) and as breeding habitat (Egea-Serrano *et al.*, 2005). However, although it has been described as being very tolerant to organic pollution (Llorente *et al.*, 2002), no studies analysing the specific effects that nitrogen compounds may have on *P. perezi* have been published.

MATERIAL AND METHODS

Five different egg masses of *P. perezi* were collected from a natural population exposed to a low level of nitrogen pollution (less than 5.1 mg $N\text{-}NO_3^-$/l (Vidal-Abarca *et al.*, 2000)) in the Segura River basin (U.T.M. 30SXH). This basin, which covers an area of 14 432 km^2 in the southeastern Iberian Peninsula, has been described as one of the most arid zones of the Iberian Peninsula (Vidal-Abarca *et al.*, 1987) and, probably, Europe (Geiger, 1973). A trend towards eutrophication of waterbodies in this basin, which comprises ponds, streams and tributaries of the main Segura River where *P. perezi* breeds, has been described (Vidal-Abarca *et al.*, 1990; Ballester, 2003).

Eggs were reared in the laboratory in 12 l aquaria, at roughly 23-25ºC, in aerated dechlorinated tap water until larvae reached the Gosner 25 developmental stage (Gosner, 1960). Then they were individually transferred to clear, food-quality, 1 l plastic beakers containing 500 ml of dechlorinated tap water. The water in the beakers was renewed every three days to prevent oxygen depletion. The larvae were fed every three days with dry dog chow pellets (250-350 mg). They reached the Gosner´s 46 developmental stage (Gosner, 1960) after 71-97 days (snout-vent length (mean ± 1 SE): 16.47 mm ± 0.53 mm, n=24). Just after reaching this developmental stage, froglets were individually transfered to 1 l plastic beakers containing 100 ml of one of the following treatments: 1) 0 mg/l (control); 2) 40 mg NH_4Cl/l; 3) 40 mg NH_4Cl/l + 500 mg $NaNO_3$/l + 100 mg $NaNO_2$/l; 4) 40 mg NH_4Cl/l + 500 mg $NaNO_3$/l + 10 mg $NaNO_2$/l. Neither eggs nor larvae were exposed to these treatments. Treatments were selected because they were observed to reduce larval survival in the aquatic phase of the studied species (Egea-Serrano *et al.*, 2009) and because the concentrations are representative of the high ammonium, nitrite and nitrate levels detected in watercourses of the Segura River basin

(e.g. 154.6 mg NH_4^+/l, 74.4 mg NO_2^-/l, 333 mg NO_3^-/l, Suárez, personal communication). Ammonium, nitrite and nitrate solutions were prepared from NH_4Cl, $NaNO_2$ and $NaNO_3$, respectively. Each beaker was randomly assigned to one of the above treatments. Treatments 1 and 3 were replicated seven times, whereas treatments 2 and 4 were replicated five times due to differences in larval survival up to metamorphosis between treatments. Although water quality was not measured in this experiment, previous tests performed under identical conditions showed that water conductivity and salinity are significantly higher in all treatments exposed to fertilizers than the control treatment (conductivity: $F_{3,54}$= 3413.769; *P*= 0.0001; salinity: $F_{3,54}$= 1340.024; *P*= 0.0001). pH showed significant differences among treatments ($F_{3,54}$= 2.878; *P*= 0.044). Nevertheless, a post hoc multiple comparison test did not confirm the existence of such differences (Tukey´s test: *P*>0.122, in all cases). No significant differences among treatments were detected in relation to water temperature ($F_{3,54}$= 0.324; *P*= 0.808)

The water in the beakers was completely renewed and the fertilizer level restored daily. The beakers were tightly closed to avoid water evaporation, ammonium volatization and the escape of any specimen. They were tilted (10º, approximately) so that half of the floor of each beaker was submerged and the other half completely dry. Froglets were observed twice daily (at 12 h intervals) over a 14 day period. Each experimental unit was observed for 30 s, recording whether the froglets were resting on the submerged half or on the emerged half (including walls) of the beakers. Observations were made at a distance of at least one metre from the experimental units to avoid disturbing the animals. Although froglets were fed *ad libitum* with flightless fruitflies, none of them ate during the duration of the experiment. No froglet died during the exposure time.

To determine whether the treatments used in the present study were avoided by the studied species, four separate statistical approaches were performed. First, to study the temporal variation during the two weeks the experiment lasted (weekly analysis) one-way repeated measures ANCOVA was used, with the number of censuses in which froglets were found in the submerged half of the beakers on each week of the experiment as dependent variable. Second, to assess the circadian variations in treatment avoidance (circadian analysis), one-way repeated measures ANCOVA was used, with the number of censuses in which froglets were found in the submerged half of the beakers in the mornings and evenings the experiment included as dependent variable. Third, to obtain an overall view (global analysis) of treatment avoidance by froglets, one-way ANCOVA was used, with the number of censuses in which froglets were found in the submerged half of the beakers over the two weeks the experiment lasted. For these three statistical analyses, the treatment was considered as independent variable and snout-vent length at metamorphosis as the covariate. Finally, the inter-individual variation in treatment avoidance was studied. Variable treatment avoidance (dependent variable) was coded as a binary variable (1: froglets on the emerged half of the beakers or on their walls; 2: froglets in the submerged half of the beakers), and differences between individuals (single factor: individual froglet) for each treatment was analysed using Kruskal-Wallis test. Additionally, repeatability (r) for each treatment was calculated using the methodology proposed by Lessells & Boag (1987). Data were transformed logarithmically (log x; log (x+1) in the case of the circadian analysis). Previous statistical analyses were performed using SPSS® statistical package v. 11.0 and a significant level of 5% was selected. Additionally, a power analysis was performed on number of censuses in which froglets were detected in the submerged half of the beakers during either the first and the second week of the experiment, mornings and

evenings included in the experiment and, finally, over the two weeks the experiment lasted using STATISTICA 6.0 statistical package (Statsoft, Inc. 2001). Since STATISTICA only performs power analyses when each group of the studied parameters shows an identical number of cases, we haphazardly selected the cases so that all treatments showed the same number of cases (n = 5). Root-mean-square error from each logarithmically transformed variable and a significant level of 5% was used.

RESULTS

The treatments were not avoided by *P. perezi* froglets as observed from weekly and circadian analyses (Table 1; Fig. 1). The global analysis showed that the presence of organic compounds in the aquatic environment does not imply its avoidance by froglets of the studied species ($F_{3,18}$= 0.124; *P*=0.945) (Table 1). In spite of these results, a significant interaction between treatment and time was detected in relation to the circadian analysis (Fig. 1). Although power was very low for all analyses performed (Table 1), the high P-values exhibited by the treatment effect (P>0.76 in all cases) suggested no support for the hypothesis concerning an effect of pollutant treatments on the avoidance response of froglets (Table 1, Fig. 1).

Froglets exposed to treatment 1 or control showed significant inter-individual variation as regards treatment avoidance (χ^2_6= 44.465, *P*= 0.0001), and the highest repeatability of all treatments (r= 0.32). Likewise, a significant effect of the individual on treatment avoidance was detected in the case of treatments 3 (χ^2_6= 28.538, *P*= 0.0001; r= 0.23) and 4 (χ^2_4= 12.858, *P*= 0.012; r= 0.11). Individuals exposed to treatment 2 showed no significant variation in treatment avoidance (χ^2_4= 5.776, *P*= 0.217) and the lowest repeatability of all treatments (r= 0.023).

Table 1. Summary statistics for the one-way repeated measures ANCOVA (weekly and circadian analysis) and one-way ANCOVA (global analysis) on treatment avoidance by froglets of *Pelophylax perezi*.

Source of variation	df	*F*	*P*
Weekly analysis (First week: 0.081; Second week: 0.089)			
Between subjects effects			
Treatment	3	0.207	0.891
SVL	1	1.299	0.269
Within subjects effects			
Time	1	0.294	0.594
Time x Treatment	3	0.046	0.986
Time x SVL	1	0.290	0.597
Error	18		
Circadian analysis (Mornings: 0.16; Evenings: 0.16)			
Between subjects effects			
Treatment	3	0.392	0.760
SVL	1	2.525	0.129
Within subjects effects			
Time	1	3.527	0.077
Time x Treatment	3	4.727	0.013
Time x SVL	1	3.393	0.082
Error	18		
Global analysis (Global: 0.056)			
Between subjects effects			
Treatment	3	0.124	0.945
SVL	1	1.184	0.291
Error	18		

SVL: snout-vent length. The result of the power analysis on the number of censuses in which froglets were detected in the submerged half of the beakers during the first and the second week of the experiment, mornings and evenings included in the experiment and over the two weeks that the experiment lasted is presented in brackets.

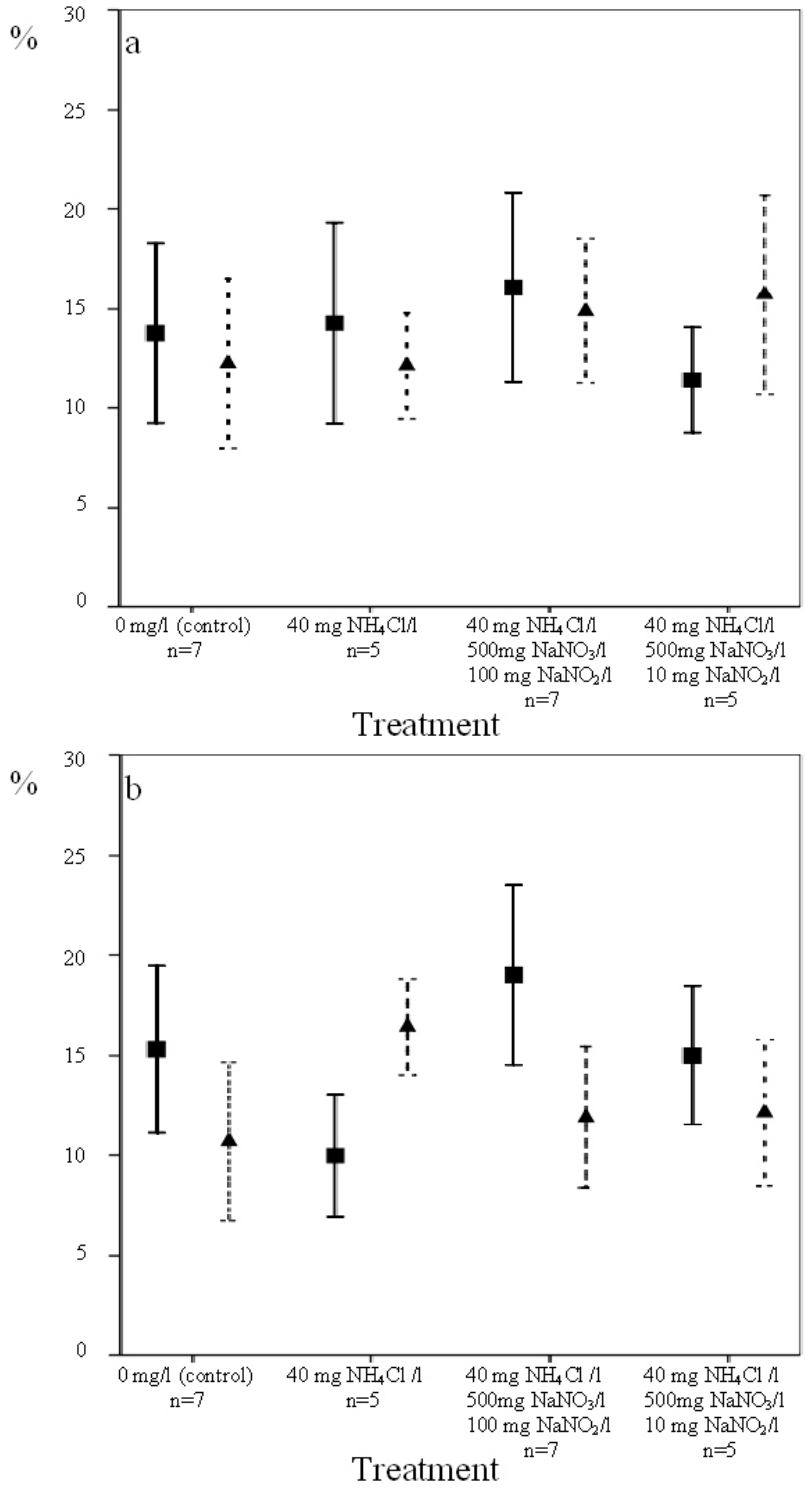

Fig. 1. Mean proportion (± 1 SE) of number of times metamorphic individuals were detected in contact with the different nitrogen water treatments for a) weekly analysis (■ first week of the experiment; ▲ second week of the experiment) and b) circadian analysis (■ mornings; ▲ evenings).

DISCUSSION

Although the power of the analyses performed was low, the results obtained suggest that none of the nitrogen polluted treatments was avoided by postmetamorphic froglets of *P. perezi*. Given that these treatments correspond to the high ammonium, nitrite and nitrate concentrations that actually occur in the Segura River basin (Suárez, personal communication), we can predict that ecologically relevant levels of eutrophication may not influence habitat use by the studied species. This highly aquatic frog uses the aquatic environment as shelter from predators (Martín *et al.*, 2006), as well as for foraging (Docampo & Vega, 1990) and breeding (Egea-Serrano *et al.*, 2005). This absence of any effect of organic concentration on habitat selection suggests that froglets are able to use the highly stressful concentrated organic habitat in spite of the detrimental consequence that this absence of avoidance may have on survival or reproductive success (Oldham *et al.*, 1997; Hatch *et al.*, 2001; Marco *et al.*, 2001; Ortiz-Santaliestra *et al.*, 2005). Nevertherless, the significant inter-individual variation in treatment avoidance, with associated moderate repeatability values, indicates that individual froglets may differ in their level of avoidance to fertilizer exposure. This could well have important effects at a populational scale since repeatability is associated with heritability placing an upper bound on heritability and thus to the potential to adapt to these stressful polluted environments (Falconer, 1989).

Previous studies have shown that different amphibian species avoid high fertilizer concentrations in their terrestrial environment in the laboratory (Hatch *et al.*, 2001; Marco *et al.*, 2001; Ortiz-Santaliestra *et al.*, 2005) and that the combination of pollutants may produce a multiplicative rather than an additive response (Brown & Spence, 2003). However, the present results suggest that postmetamorphic froglets of *P.*

perezi do not exhibit an avoidance response to single and combined fertilizer stressors. Marco *et al.* (2001) suggested that the vulnerability of amphibian postmetamorphic stages to fertilizers could be due to nitrogen uptake through their permeable skin. In aquatic and semiaquatic anurans, pulmonary ventilation is more important than cutaneous gas exchange and, as a consequence, their skin is less vascularized than that of terrestrial anurans (Duellman & Trueb, 1994). Considering the aquatic habits of *P. perezi* (Lizana *et al.*, 1989), its cutaneous vascularization may not be highly developed, which would make its skin scarcely permeable. So, these characteristics may confer a certain degree of tolerance to fertilizers. In addition, Hatch *et al.* (2001) attributed the non- avoidance of high urea concentration in soil substrate by juvenile *Bufo boreas* to the stress that toads could have experienced during the experiment, which may have prevented them from detecting such concentration. Ammonium can be transformed into ammonia, a toxic nitrogen compound for amphibians (Jofre & Karasov, 1999), which can volatilize (Ritter & Bergstrom, 2001), entering the amphibians through the skin and lungs. Although treatments were renewed daily, it is possible that the beakers containing the fertilizer treatments accumulated an ammonia concentration sufficiently high to stress the froglets, preventing them from detecting the presence of fertilizers and from avoiding them. Taking into account these considerations, the lack of habitat avoidance could be explained by both a species tolerance to fertilizers as well as by a sublethal response to them. Thus, no conclusive explanation can be provided. Given that a species may avoid low fertilizer concentrations but not higher levels (Hatch *et al.*, 2001), future research considering fertilizer concentrations differing from those employed in the present study are needed to determine whether *P. perezi* is tolerant to fertilizers or whether it can detect and avoid their presence in the environment.

The general avoidance of water by froglets detected in the present study is of note. Taking into consideration that the beakers were closed, this result cannot be attributed to toxic fumes from surrounding beakers which could affect all froglets. On the contrary, this avoidance could be explained by considering the results presented by Lizana *et al.* (1989), who described that postmetamorphic individuals are forced to disperse from ponds where they developed. So, although a more specific study on habitat selection by postmetamorphic *P. perezi* has not been performed and, consequently, any explanation would be hypothetical, this result might suggest that froglets avoid water even in laboratory conditions as a consequence of an endogenous trend to disperse to land, as it occurs in the wild (Lizana *et al.*, 1989). This consideration emphasizes the relevance of developing future studies about treatment avoidance with adult *P. perezi* to test whether this developmental stage is more aquatic than the juvenile stage and whether it is more affected by the presence of nitrogenous compounds in the water.

Finally, it must be noted that it is difficult to generalize to the field the results obtained from laboratory experiments. This study suggests that nitrogen pollution has no influence on the habitat use of *P. perezi* froglets. Nevertheless, considering that the substrate can affect fertilizer avoidance behavior (Hatch *et al.*, 2001), and that permanent waterbodies inhabited by this species (Díaz-Paniagua, 1990), as well as the soil surrounding them, may be polluted by the presence of different nitrogenous compounds, pesticides and their degradates, the behaviour of the studied species in the field could differ significantly from the results presented in this work. So, studies representing a more realistic approach to natural conditions are indispensable for assessing the actual impact fertilizer pollution has on *P. perezi* habitat use.

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BLOQUE III

ANÁLISIS DE LOS EFECTOS DE LOS COMPUESTOS NITROGENADOS EN *PELOPHYLAX PEREZI* EN EXPERIMENTOS DE MESOCOSMOS Y CAMPO

CAPÍTULO 7

EFFECTS OF NITROGENOUS POLLUTION ON SURVIVAL, MORPHOLOGY AND GROWTH OF LARVAE OF THE IBERIAN WATERFROG, *PELOPHYLAX PEREZI* (SEOANE, 1885): A HIERARCHICAL APPROACH TO NATURAL CONDITIONS

Abstract. Pollution has been linked to the decline of amphibians. However, most of the studies dealing with the impact of pollutants on amphibians have been carried out in the laboratory, being necessary further research to determine whether the information available ensue in natural conditions. Thus, we assessed the impact of the exposure to NH_4Cl (isolated or combined with $NaNO_2$ and $NaNO_3$) and to natural streams differing in their degree of pollution on fitness-related larval traits for three populations of *Pelophylax perezi* exposed to different degrees of eutrophication in two different experiments carried out in mesocosms and enclosures. The results obtained indicate that in mesocosm conditions larval mortality was unaffected by treatments and that in such conditions the exposure to the combination of nitrogenous compounds reduced body and tail depth and final mass and growth. Paradoxically, in more natural conditions, the exposure to polluted localities increased larval mortality, size, final mass and growth. Moreover, although population-specific responses were observed in mesocosms for the response variables tail length and depth, tail muscle width and final mass, they were not detected when larvae were exposed to natural field sites. These results point out that, although the study species can be negatively affected by the exposure to nitrogenous compounds, it is quite tolerant to nitrogenous pollution in the wild. We suggest that the results recorded in enclosure experiments should be accounted for when assessing the actual impact of pollutants on amphibians.

Key words: Nitrogenous pollution, inter-populational variation, *Pelophylax perezi*, larvae, natural conditions

INTRODUCTION

Amphibian population decline has been described as a global phenomenon (Gardner, 2001; Blaustein & Kiesecker, 2002). Although natural fluctuations may cause demographic variation (Pechmann *et al.*, 1991; Tejedo, 2003), the role of anthropogenic influence on such decline has been recognized (Scoccianti, 2001, Semlitsch, 2003). Thus, aspects dealing with overexploitation, habitat loss, disease, pollution and climatic change have linked to amphibian population degradation (Collins & Storfer, 2003; Stuart *et al.*, 2004). As regards environmental chemical pollution, this factor has been emphasized as one of the causes of amphibian declines (Scoccianti, 2001, Semlitsch, 2003). Some studies related such decline to the upwind of agriculture in different regions of the world (Berger, 1989; Davidson *et al.*, 2001,2002; Hamer *et al.*, 2004). Since farming practices may increase the concentration of nitrogenous compounds in the environment (Ritter & Bergstrom, 2001), the study of the effects of such compounds is of great relevance, specially considering that their presence in the environment is widespread (Carpenter *et al.*, 1998) and is expected to increase in the future (Tilman *et al.*, 2001; Galloway *et al.*¸ 2003).

The efforts to assess the impact of nitrogenous compounds on amphibians are growing (see review Marco & Ortiz-Santaliestra, in press). Both lethal and sublethal effects on amphibian larvae have been reported for ammonium, nitrite and nitrate (e.g. Xu & Oldham, 1997; Marco *et al.*, 1999; Griffis-Kyle, 2007; Egea-Serrano *et al.*¸ 2009). Nevertheless, the great inter- (Marco *et al.* 1999) and intraspecific variation (Johansson *et al.*, 2001; Egea-Serrano *et al.*, 2009) in relation to the tolerance to nitrogenous compound exposure makes of great relevance to develop further researches to complete the existing database (Smith *et al.*, 2005).

The exposure to cocktails of stressing factors may exarcerbate their effects of such factors acting isolated through additive or synergistic responses (Berenbaum, 1989). The combination of fertilizers with other stressing factors such as pesticides (Boone *et al.*¸ 2005), UV-B radiation (Hatch & Blaustein, 2000, 2003; Macías *et al.*, 2007) or low pH (Hatch & Blaustein, 2000) may affect amphibian larvae more severely than when they act isolated. Nevertheless, in spite of the relevance of integrating multiple stressing factors, most ecotoxicological studies deal with the effects of a single pollutant (Storfer, 2003). In natural environments, nitrogenous compounds are present in combination with complex cocktails of other factors, including other nitrogenous compounds (e.g. Vidal-Abarca *et al.*, 2000). However, the number of studies analysing the impact that different combinations of such compounds may have on amphibian larvae is very scarce (Egea-Serrano *et al.*, 2009).

Most of the studies dealing with amphibian ecotoxicology have been performed in laboratory conditions (Boone & James, 2005). Although data collected in these conditions are a starting point in understanding the effects of a pollutant, it is necessary to determine whether the effects recorded in the laboratory ensue in the field (Boone & Bridges, 2003; Boone & James, 2005). Pond mesocoms and field enclosures have been described as useful tools to document the effect of pollutants in realistic conditions (Boone & Bridges, 2003; Boone & James, 2005). Mesocosms have been defined as independent, closed outdoor artificial systems (either aquatic or terrestrial) containing food webs and processes representative of natural environment whereas enclosures are defined as permeable containers enclosing the study organisms within a particular environment, allowing environmental exchange among enclosures (Boone & James, 2005). The characteristics of these methodological approaches allow to study properly

population- and community level processes as well as to integrate multiple stressing factors in naturally changing environments (Boone & James, 2005).

The aims of the present study were 1) to determine the effects of high concentration of ammonium chloride, isolated and combined with sodium nitrite and sodium nitrate, on mortality, morphology, weight and growth of larvae of *Pelophylax perezi* exposed in mesocom condition (common garden experiment); 2) to determine the effects of the real exposure to toxicants by employing enclosures in streams differing in their level of nitrogenous pollution (field experiment) on mortality, morphology, weight and growth of larvae; and, 3) to evaluate whether differences among populations in their tolerance to nitrogenous pollution exists. *P. perezi* widespreads through the Iberian Peninsula and Southern France (Llorente & Arano, 1997). It inhabits mainly permanent water bodies (Díaz-Paniagua, 1990). These habitats, as a consequence of farming practices (one of the most important nitrogen sources in the nature (e.g. Ritter & Bergstrom, 2001), may hold high concentrations of different nitrogen forms (e.g. for southeastern Iberian Peninsula: 154.6 mg NH_4^+/l; 74.4 mg NO_2^-/l; 333.0 mg NO_3^-/l, Suárez, personal communication). So, this species might be exposed to and threatened by nitrogen pollution through all its life cycle, because it uses water bodies as shelter from predators (Martín *et al.*, 2006) and as foraging (Docampo & Vega, 1990) and breeding habitat (Egea-Serrano *et al.*¸ 2005). On the other hand, the wide distribution range of *P. perezi* makes possible the existence of interpopulational variation in tolerance to different levels of nitrogenous pollution, as it has been shown in other studies (Johansson *et al.*, 2001; Egea-Serrano *et al.*, 2009). This consideration, together with the existence of different nitrogenous ions in the field which may act jointly, points out the relevance of analysing the interpopulational variation and the effect of

nitrogenous cocktails in environmentally representative conditions to accurately assess the impact of nitrogenous pollution on a widespread distributed amphibian species.

MATERIAL AND METHODS

Studied populations

Five different egg masses of *P. perezi* were collected from three populations located in the Segura River Basin in the first fortnight of March 2007. This basin has been reported as one of the most arid of Iberian Peninsula (Vidal-Abarca *et al.*, 1987), and the water bodies located in it has been described to show a trend towards eutrophication (Ballester, 2003). The selected populations were naturally exposed to highly different levels of nitrogen pollution. Two of these populations corresponded to the permanent headwater stream Río Chícamo (38º12´N, 001º03´W; 170.3 m.a.s.l.) and to a seminatural pond located in the Sierra Espuña Regional Park (37º52´N, 001º30´W; 673.0 m.a.s.l.) (C1 and C2, hereafter). C1 showed low nutrient concentration (less than 5.1 mg $N\text{-}NO_3^-$/l (Vidal-Abarca *et al.*, 2000)). Although no data about nutrient concentration is available for C2, it is unlike that amphibians were exposed to pollution because in the surroundings of this locality neither urban nor farming activities are present. The surrounding terrestial environment of these populations corresponds to bush on marls (C1) or to pine trees on limestone lithology (C2). In contrast to the previous populations, the third one is located in another semipermanent headwater stream, Rambla del Garruchal (37º57´N, 001º04´W; 346.0 m.a.s.l.) (herafter P1), which has been exposed at least for the last 22 years to nitrate concentration as high as 162.1 mg NO_3^-/l (Ballester, 2003) due to intensive farming activities and subsequent run-off in its basin. In addition, its natural environment corresponds with pine trees on

heterogeneous carbonated materials. However, through most of the course of the stream the habitat has been largely modified being dominated by intensive cattle exploitations. The geographical distance between populations ranged from 28.3 km to 54.9 km.

Experimental design and response variables

Developmental stage of embryos when they were collected ranged from 15 to 18 Gosner´s stage (Gosner, 1960), not existing differences among populations in developmental stage (Chi-square, $P > 0.05$). Embryos were reared in 12 l glass aquaria containing dechlorinated tap water (pH = 8.39; conductivity = 985 µS/cm; 0.002 mg NO_2^-/l; 4.69 mg NO_3^-/l). When they reached Gosner´s 25 developmental stage, they were exposed to treatments.

Common garden experiment.- A total of 16 plastic pools (430 l of capacity) were prepared in an outdoor facility at the Campus Universitario de Espinardo (Universidad de Murcia) one month prior to the beginning of the experiment. They were filled with 200 l of dechlorinated tap water. Once a week before the experiment began, each pool was inoculated with 0.5 l of water from a natural pond (Boone *et al.*¸ 2004). Since this pond (37º52´N, 001º34´W; 1124 m.a.s.l.) is located within a pine forest in a protected area of Southern Spain, it is unlikely that the water used to inoculate the pools was exposed to pollution. In the moment of the beginning of the experiment (26 March 2007), pools had a thin leaf litter that provided natural feeding resources to tadpoles and contributed to make nutrient dynamics more natural. Prior to placing the larvae in the pools, water volume in each pool was reduced to 150 l, to correct for differences in water volume due to evaporation. Additionally, when larvae were placed in the pools, each tank was equipped with a plastic tile (229 cm^2) facing south to assess periphyton biomass (Relyea *et al.*, 2005). Five larvae from the three study sites were located in

each pond. Since larvae used in this experiment came from different populations, larvae mixing prevention was essential to preserve populational identity. Therefore, each larvae was individually placed in 1l plastic beaker covered with 1 mm mesh lid within each pool. Each pool contained five larvae from each population. Initial mass of larvae did not differed in relation to their population of origin ($F_{2, 237} = 1.064$; $P = 0.347$): C1 (mean ± 1 SE): 0.043 g ± 0.002 g, n = 80; C2 (mean ± 1 SE): 0.046 g ± 0.002 g, n = 80; P1 (mean ± 1 SE): 0.041 g ± 0.002 g, n = 80.

Larvae were acclimatized in the pools two days before the beginning of the experiment. At this moment, one dog chow pellet (250-350 mg) was placed in each beaker. No additional food was provided to larvae during the experiment. Each pool was haphazardously assigned to one of the four following treatments: 1) control; 2) 13.5 mg NH_4^+/l; 3) 13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 6.67 mg NO_2^-/l; 4) 13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 66.7 mg NO_2^-/l. These treatments were selected because they produced the highest larval mortality in the laboratory (Egea-Serrano *et al.*, 2009) and concentrations were within those naturally occurring in the field in the Segura River basin (e.g. 154.6 mg NH_4^+/l; 74.4 mg NO_2^-/l; 333.0 mg NO_3^-/l, Suárez, personal communication). To obtain the experimental concentrations, 40 g NH_4Cl/l, 70 g $NaNO_2$/l and 150 g $NaNO_3$/l stock solutions were pipetted directly into the pools. Each treatment was replicated four times. The experiment thus consisted in a split-plot design (Quinn & Keough 2002), where treatment was the main plot factor and the population of origin the subplot factor.

Field experiment.- Larvae from the three study populations were reared in four selected natural streams. Two of them, Rambla del Estrecho (37º46´N, 001º45´W; 476.7 m.a.s.l.) and Rambla Tejera (38º11´N, 002º07´W; 1197.9 m.a.s.l.), correspond, respectively, to permanent and semipermanent headwater streams located in forest

natural environments, which makes unlikely the presence of nitrogenous pollution in the water column. During the exposure period nitrogenous compounds levels were (mean ± SE): Rambla del Estrecho: 0.6 mg NO_3^-/l ± 0.08, n = 3; Rambla Tejera: 1.4 mg NO_3^-/l ± 0.3, n = 4). The rest of the destination sites, Río Quípar (38º02´N, 001º54´W; 710 m.a.s.l.) and Río Mula (38º03´N, 001º25´W; 190.2 m.a.s.l.) corresponds to permanent rivers exposed to high degree of eutrophication as a consequence of urban wastewaters and farming practices. During the exposure period their nitrogenous compounds levels were (mean ± SE): Río Quípar: 25.02 mg NO_3^-/l ± 3.0, n = 4; Río Mula: 10.1 mg NO_3^-/l ± 1.1, n = 4). The natural presence of *P. perezi* was confirmed in all of these streams.

Eight larvae from the three studied populations were placed at each experimental locality. Initial mass of larvae did not differed in relation to their population of origin ($F_{2,69}$= 1.936; P= 0.152): C1 (mean ± 1 SE): 0.033 g ± 0.002 g, n = 72; C2 (mean ± 1 SE): 0.030 g ± 0.003 g, n = 72; P1 (mean ± 1 SE): 0.029 g ± 0.003 g, n = 72. To preserve an estimation of interpopulational variation, each larvae was individually placed in 1.5 l plastic beakers covered with 1 mm mesh lid. Larvae from each population were haphazardously distributed among three different enclosures; each one separated at least 1 m of the others. Larvae were fed with one dog chow pellet (250-350 mg) at the beginning of the experiment. No food was additionally provided during the rest of the experiment. All larvae exposed to Rambla Tejera died as a consequence, probably, of the increase of water turbidity occurred after heavy rains that took place during the experiment. Thus, this locality was removed from the subsequent statistical analyses. Therefore, the design for the field experiment consisted of one control site (Rambla del Estrecho, L1 hereafter) and two polluted sites (Río Quípar and Río Mula, hereafter L2 and L3, respectively).

For both common garden experiment and field experiment, larval mortality and mass of surviving larvae (±0.0001 g) were registered after 21 days of exposure. Since data regarding initial and final larval mass were recorded, larval growth was calculated (final mass-initial mass). Additionally seven morphological traits were recorded (mm): body length (BL), width (BW) and depth (BD); tail muscle width (TMW) and depth (TMD); tail length (TL) and depth (TD) (Fig. 1). To do so, digital images were obtained for each larvae, which were measured with the software Image-Pro Plus version 4.5.0.29 for Windows.

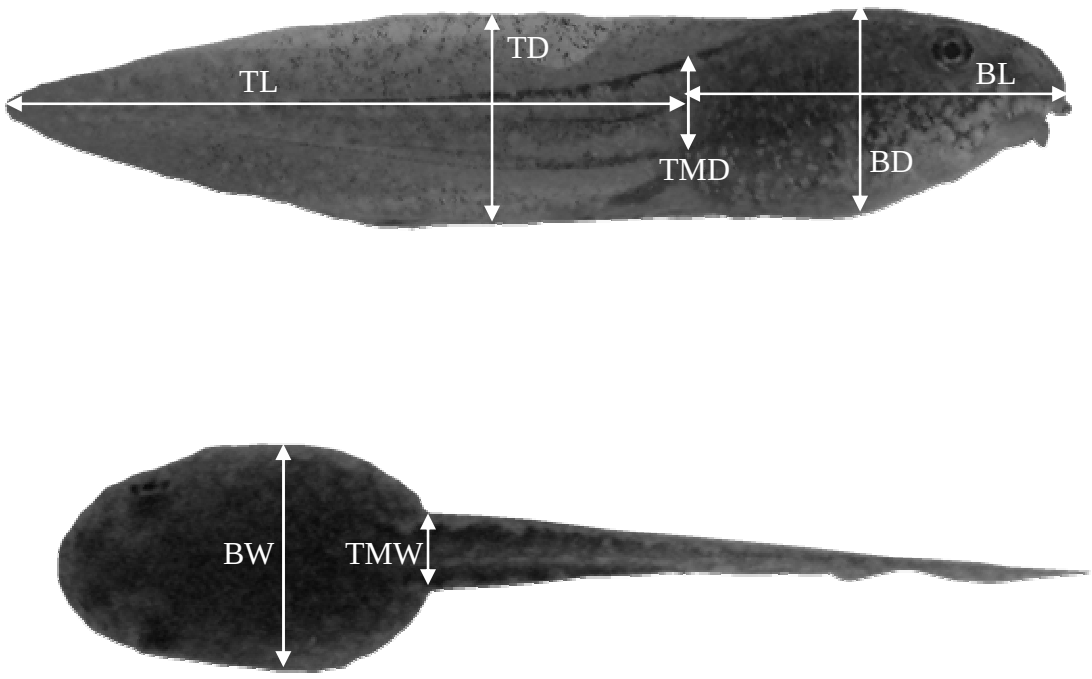

Fig. 1. Tadpole morphological measures. BL: body length; BW: body width; BD: body depth; TMW: tail muscle width; TMD: tail muscle depth; TL: tail length; TD: tail depth.

On days 2 (day 0 for the field experiment), 7, 14 and 21 after the begining of the experiment, water physicochemical characteristics (pH, temperature [ºC], conductivity [µS/cm], salinity [g/l] and depth [cm]) were measured in each pool and field localities. At the same time, a water sample was taken from each pool and field locality to establish the ammonium, nitrite and nitrate concentrations in the water column. Ammonium and nitrite analyses were performed by colorimetric methods whereas nitrate concentration was estimated by ionic chromatography. Additionally, periphyton biomass at the end of the experiment was measured for each pool to estimate a supplement resource to the tadpoles that may eventually affect their growth and perfomance (Boone *et al.*, 2005, 2007). Periphyton biomass was determined by scratching, inmediately after the experiment finished, the top surface of peryphiton tiles. Once the peryphiton obtained was dried allowing the waper to evaporate, it was weighed (± 0.0001 g). In the case of the field experiment periphyton biomass was not enough to be measured accurately.

For the common garden and field experiments, water physicochemical variables, nitrogenous ion concentration and final periphyton biomass (only for the common garden experiment) were analysed by ANOVAs. Treatment and time of measurement (only for physicochemical variables) were considered as fixed factor and pool as random factor nested within treatment. However, due to shortage of samples with detectable values for the nitrogenous ion concentrations, both in the mesocosm and field experiments, data belonging to different tanks or enclosure were pooled, which made time of measurement unable to be estimated and was not considered in our design. Because periphyton biomass was estimated only at the end of the common experiment, this factor was also excluded from the statistical analysis of this variable. Additionally, since the nutrient concentration and water physicochemistry estimates for the field

experiment were taken outside enclosures, the factor enclosure (nested within locality) was not considered when analysing physicochemical and nutrient data.

Larval mortality was analysed by generalized nonlinear models (GLZ) for binary data using STATISTICA 6.0 statistical package (Statsoft, Inc. 2001), including population of origin, treatment and pool or enclosure (nested within treatment or locality, respectively) as factors, and initial mass (log-transformed) as covariate.

Larval morphology was analysed employing absolute data. Each dependent variable was analysed by ANCOVAs, where population of origin and treatment (or locality) were fixed factor and pool or enclosure (nested within treatment or locality to correct for the spatial heterogeinity in the experiment) were random factor. Considering the hypothesis suggesting that final mass, growth and morphology may be affected by both initial size (since it is correlated with egg size and, thus, of maternal induced effect [Kaplan & Phillips, 2006]) and final size, initial and final mass were included in separate analyses to correct for such influences. All variables were log-transformed.

Statistical analyses of morphology, mass and growth were performed using the statistical software SPSS® v. 15.0.

RESULTS

Common garden experiment

Water physicochemistry

Water physicochemical variables significantly differed throughout time, which also affected the effect of treatments on such parameters, with the exception of water depth and temperature (Table 1; Fig. 2). Water physicochemical characteristics (with the exception of temperature and depth) were significantly affected by treatments (Table 1). Treatments corresponding to the combination of different nitrogenous compounds showed higher values for pH than control treatment, as well as higher conductivity and salinity than the rest of treatments (Fig. 2). Furthermore, the time x treatment interaction indicates that the differences were increased with time for pH and reduced for conductivity and salinity (Table 1; Fig. 2).

Polluted treatments significantly increased ammonium, nitrite and nitrate concentrations present in the water column in relation to control treatment (Table 2; Fig. 3). Moreover, in the case of nitrite and nitrate, the combination of nitrogenous compounds increased their concentration in relation to the rest of treatments (Fig. 3).

Table 1. Summary statistics of repeated measures ANOVAs on physicochemical characteristics of water in the experimental pools for the common garden experiment. ndf: numerator degrees of freedom; ddf: denominator degrees of freedom.

Variable	Source of variation	ndf	ddf	F	P
pH	Treatment	3	12	6.325	0.008
	Pool(Treatment)	12	36	1.569	0.145
	Time	3	36	5.769	0.003
	Time x Treatment	9	36	2.442	0.028
Temperature	Treatment	3	12	1.011	0.422
	Pool(Treatment)	12	36	0.841	0.610
	Time	3	36	87.572	0.0001
	Time x Treatment	9	36	1.106	0.384
Conductivity	Treatment	3	12	54.163	0.0001
	Pool(Treatment)	23	36	1.968	0.058
	Time	3	36	180.172	0.0001
	Time x Treatment	9	36	12.072	0.0001
Salinity	Treatment	3	12	37.539	0.0001
	Pool(Treatment)	12	36	2.101	0.043
	Time	3	36	125.290	0.0001
	Time x Treatment	9	36	5.232	0.0001
Depth	Treatment	3	12	0.366	0.779
	Pool(Treatment)	12	36	7.864	0.0001
	Time	3	36	338.351	0.0001
	Time x Treatment	9	36	0.419	0.916

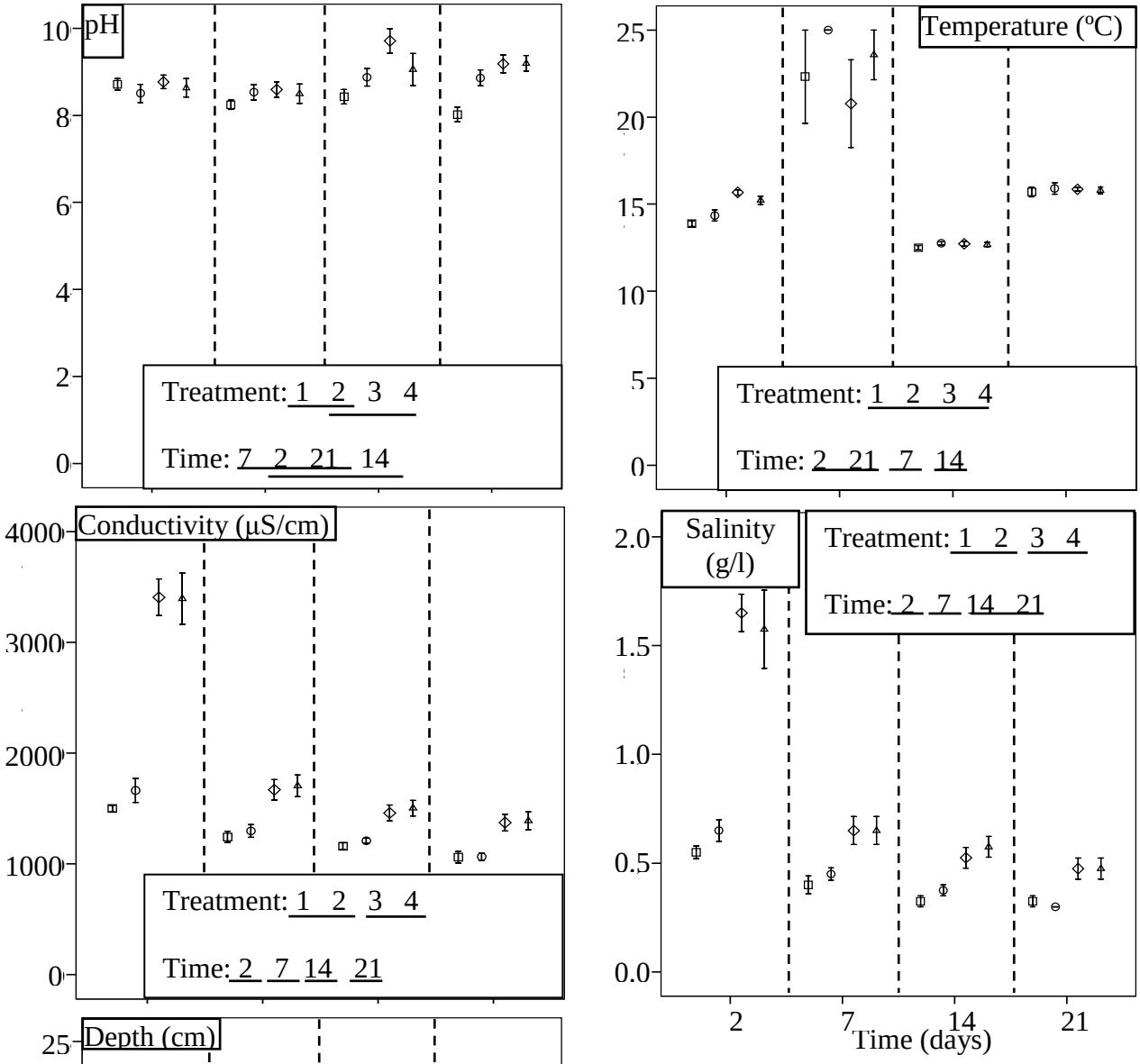

Fig. 2. Mean (±1 SE) for water physicochemical characteristics in the experimental pools. Results of HDS Tukey´s test (α= 5%) performed for the factor treatment and time of measurement are shown (common underbars notation). Treatments: □ l ;◯ 2;◇ 3;△ 4. 1) control; 2) 13.5 mg NH_4^+/l; 3) 13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 6.67 mg NO_2^-/l; 4) 13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 66.7 mg NO_2^-/l

Table 2. Summary statistics of ANOVAs on nitrogenous ion concentration in the experimental pools for the common garden experiment. ndf: numerator degrees of freedom; ddf: denominator degrees of freedom.

Variable	Source of variation	ndf	ddf	F	P
Ammonium	Treatment	3	13.014	15.111	0.0001
	Pool(Treatment)	12	36	0.538	0.875
Nitrite	Treatment	3	11.980	19.410	0.0001
	Pool(Treatment)	11	34	3.060	0.006
Nitrate	Treatment	3	12.927	68.516	0.0001
	Pool(Treatment)	12	35	1.693	0.111

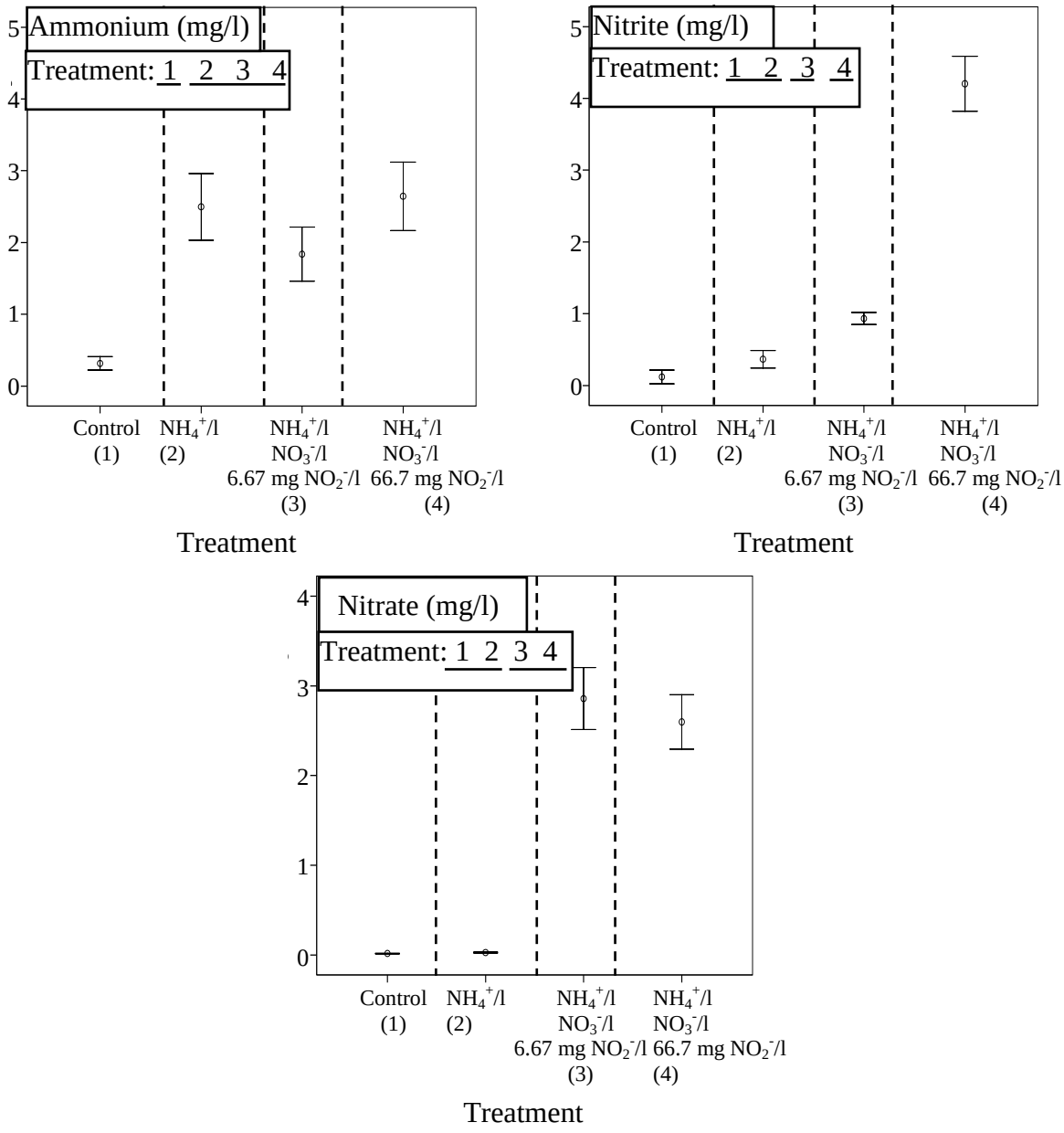

Fig. 3. Mean (±1 SE) for ammonium, nitrite and nitrate concentrations (mg/l) in the experimental pools. Treatment codes are shown in brackets. Results of HDS Tukey´s test (α = 5%) performed for the factor treatment are shown (common underbars notation).

Periphyton biomass

Growth of periphyton in the pools was significantly affected by treatments ($F_{3,12}$=23.341; P= 0.0001), being higher for those pools exposed to nitrogenous compounds (Fig. 4).

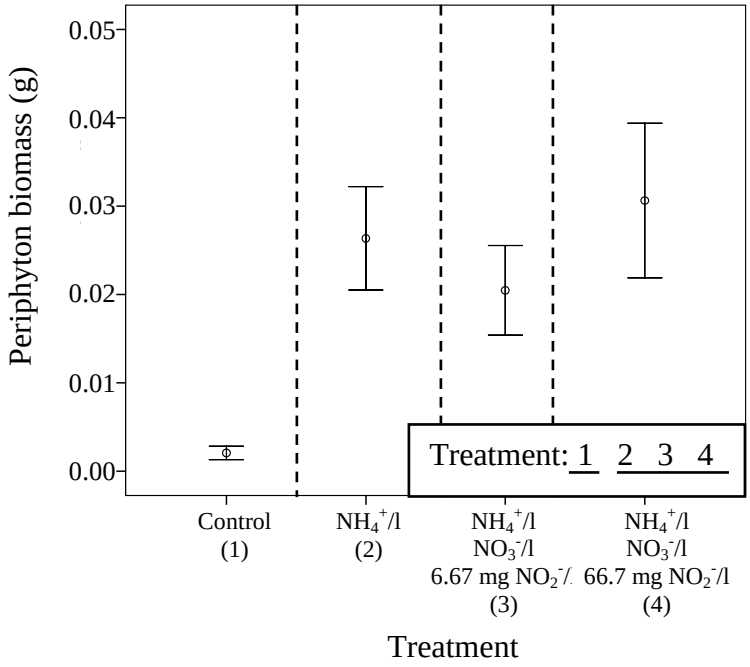

Fig. 4. Mass (mean ± 1 SE) of periphyton grown in the experimental tanks after 21 days of exposure to different levels of nitrogenous pollution. Treatment codes are shown in brackets. Results of HDS Tukey´s test (α= 5%) performed for the factor treatment are shown (common underbars notation).

Larval mortality

Neither treatments nor population of origin affected mean larval mortality (Table 3; Fig. 5). The interaction between population of origin and treatment did not reveal the existence of interpopulational differences in relation to larval tolerance to treatments (Table 3).

Table 3. Summary statistics of GLZ for binary data for larval mortality after 21 days of exposure in the common garden experiment. df: degrees of freedom. NA: not applicable.

	Not correcting for initial mass				Correctig for initial mass			
Source of variation	df	Log-likelihood	X^2	*P*	df	Log-likelihood	X^2	*P*
Population of origin	2	-60.268	2.243	0.326	2	-60.268	2.243	0.326
Treatment	3	-58.639	3.258	0.353	3	-58.639	3.258	0.353
Pool(Treatment)	12	-49.853	17.571	0.129	12	-49.853	17.571	0.129
Population x Treatment	6	-47.414	4.878	0.560	6	-47.414	4.878	0.560
Initial mass	NA	NA	NA	NA	1	-47.157	0.514	0.473

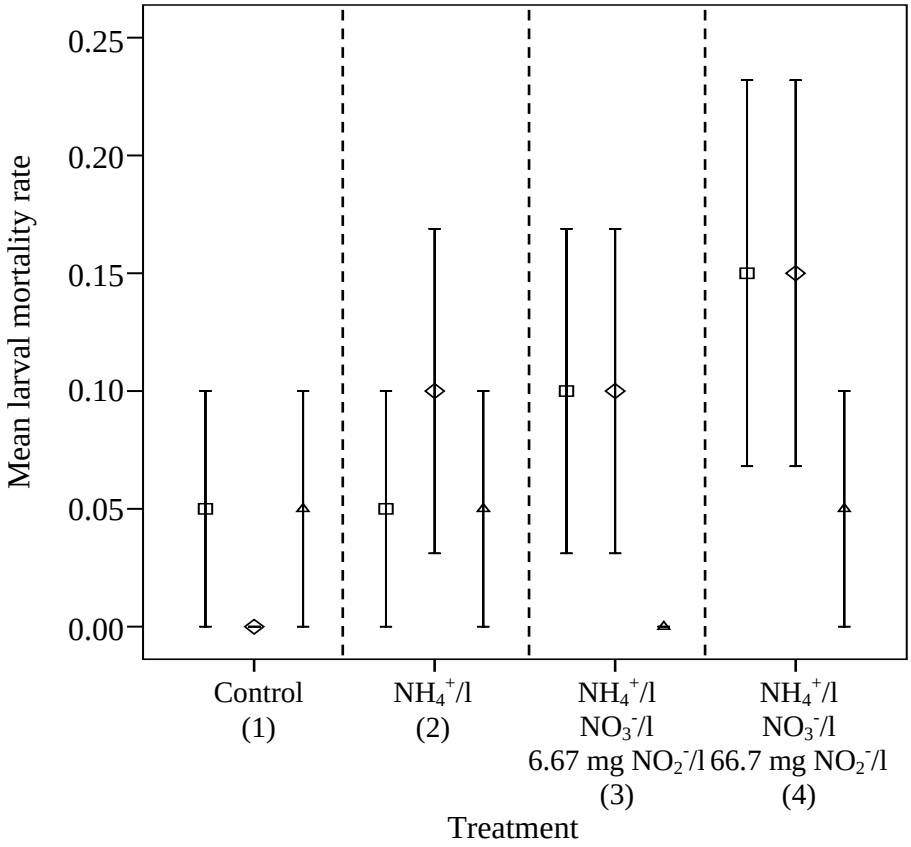

Fig. 5. Mean (± 1 SE) mortality of larvae of *Pelophylax perezi* exposed to different nitrogenous treatments in the common garden experiment. Treatment codes are shown in brackets. □ C1; ◇ C2; △ P1.

Larval morphology

Larvae exposed to nitrogenous compounds showed a consistent trend to have lower mean values for the morphological traits analysed than control larvae (Fig. 6). Nevertheless, when data were corrected by initial mass or final mass, such effect disappears to great extent (Table 4). For $\alpha = 5\%$, only body and tail depth were affected by treatment when they were corrected for initial mass. Population affected all morphological variables, being mean values higher for larvae from C2 population (Fig. 6). Additionally, we found some significant interactions between population of origin and treatment, all of them concerning tail morphology (Table 4; Fig. 6).

Table 4. Summary statistics of ANCOVAs performed on the morphological traits of tadpoles after 21 of exposure in the common garden experiment. All variables were log-transformed. * P<1%; ** P<5%; ***P<10%. NA: not applicable.

Variable	Source of variation	Not correcting for mass	Correcting for initial mass	Correcting for final mass
BL	Population	$F_{2,196}$= 5.877*	$F_{2,195}$= 5.552*	$F_{2,195}$= 1.893
	Treatment	$F_{3,12.010}$= 3.081***	$F_{3,12.043}$= 3.140***	$F_{3,14.746}$= 0.680
	Mass	NA	$F_{1,195}$= 0.850	$F_{1,195}$= 114.219*
	Pool(Treatment)	$F_{12,196}$= 11.990*	$F_{12,195}$= 11.838*	$F_{12,195}$= 1.608***
	Population x Treatment	$F_{6,196}$= 1.716	$F_{6,195}$= 1.731	$F_{6,195}$= 1.258
BD	Population	$F_{2,196}$= 9.925*	$F_{2,195}$= 9.530*	$F_{2,195}$= 6.002*
	Treatment	$F_{3,12.030}$= 4.132**	$F_{3,12.135}$= 4.169**	$F_{3,15.338}$= 0.426
	Mass	NA	$F_{1,195}$= 0.188	$F_{1,195}$= 73.944*
	Pool(Treatment)	$F_{12,196}$= 3.860*	$F_{12,195}$= 3.798*	$F_{12,195}$= 1.336
	Population x Treatment	$F_{6,196}$= 1.802	$F_{6,195}$= 1.791	$F_{6,195}$= 1.512
BW	Population	$F_{2,196}$= 11.490*	$F_{2,195}$= 10.553*	$F_{2,195}$= 5.634*
	Treatment	$F_{3,12.030}$= 2.279	$F_{3,12.138}$= 2.560	$F_{3,15.540}$= 3.204***
	Mass	NA	$F_{1,195}$= 2.106	$F_{1,195}$= 64.072*
	Pool(Treatment)	$F_{12,196}$= 3.849*	$F_{12,195}$= 3.707*	$F_{12,195}$= 1.264
	Population x Treatment	$F_{6,196}$= 1.158	$F_{6,195}$= 1.348	$F_{6,195}$= 1.024
TL	Population	$F_{2,196}$= 22.716*	$F_{2,195}$= 22.806*	$F_{2,195}$= 11.444*
	Treatment	$F_{3,12.011}$= 2.259	$F_{3,12.048}$= 2.115	$F_{3,14.973}$= 0.179
	Mass	NA	$F_{1,195}$= 0.363	$F_{1,195}$= 88.347*
	Pool (Treatment)	$F_{12,196}$= 10.659*	$F_{12,195}$= 10.653*	$F_{12,195}$= 1.491
	Population x Treatment	$F_{6,196}$= 1.819***	$F_{6,195}$= 1.586***	$F_{6,195}$= 1.808***

Table 4 (continued). Summary statistics of ANCOVAs performed on the morphological traits of tadpoles after 21 of exposure in the common garden experiment. All variables were log-transformed. * P<1%; ** P<5%; ***P<10%. NA: not applicable.

Variable	Source of variation	Not correcting for mass	Correcting for initial mass	Correcting for final mass
TD	Population	$F_{2,196}$= 9.535*	$F_{2,195}$= 9.851*	$F_{2,195}$= 2.024
	Treatment	$F_{3,12.018}$= 4.651**	$F_{3,12.076}$= 4.292**	$F_{3,15.386}$= 0.307
	Mass	NA	$F_{1,195}$= 0.773	$F_{1,195}$= 101.675*
	Pool(Treatment)	$F_{12,196}$= 6.686*	$F_{12,195}$= 6.736*	$F_{12,195}$= 1.318
	Population x Treatment	$F_{6,196}$= 2.774**	$F_{6,195}$= 2.754**	$F_{6,195}$= 2.029***
TMD	Population	$F_{2,196}$= 17.411*	$F_{2,195}$= 16.687*	$F_{2,195}$= 8.239*
	Treatment	$F_{3,12.010}$= 2.573	$F_{3,12.044}$= 2.607	$F_{3,14.047}$= 0.547
	Mass	NA	$F_{1,195}$= 0.501	$F_{1,195}$= 56.580*
	Pool(Treatment)	$F_{12,196}$= 11.667*	$F_{12,195}$= 11.506*	$F_{12,195}$= 2.133**
	Population x Treatment	$F_{6,196}$= 1.326	$F_{6,195}$= 1.332	$F_{6,195}$= 1.078
TMW	Population	$F_{2,196}$= 31.676*	$F_{2,195}$= 31.475*	$F_{2,195}$= 20.433*
	Treatment	$F_{3,12.012}$= 2.873***	$F_{3,12.051}$= 2.733***	$F_{3,13.170}$= 0.712
	Mass	NA	$F_{1,195}$= 0.165	$F_{1,195}$= 31.139*
	Pool(Treatment)	$F_{12,196}$= 10.092*	$F_{12,195}$= 10.060*	$F_{12,195}$= 3.673*
	Population x Treatment	$F_{6,196}$= 2.612**	$F_{6,195}$= 2.514**	$F_{6,195}$= 2.236**

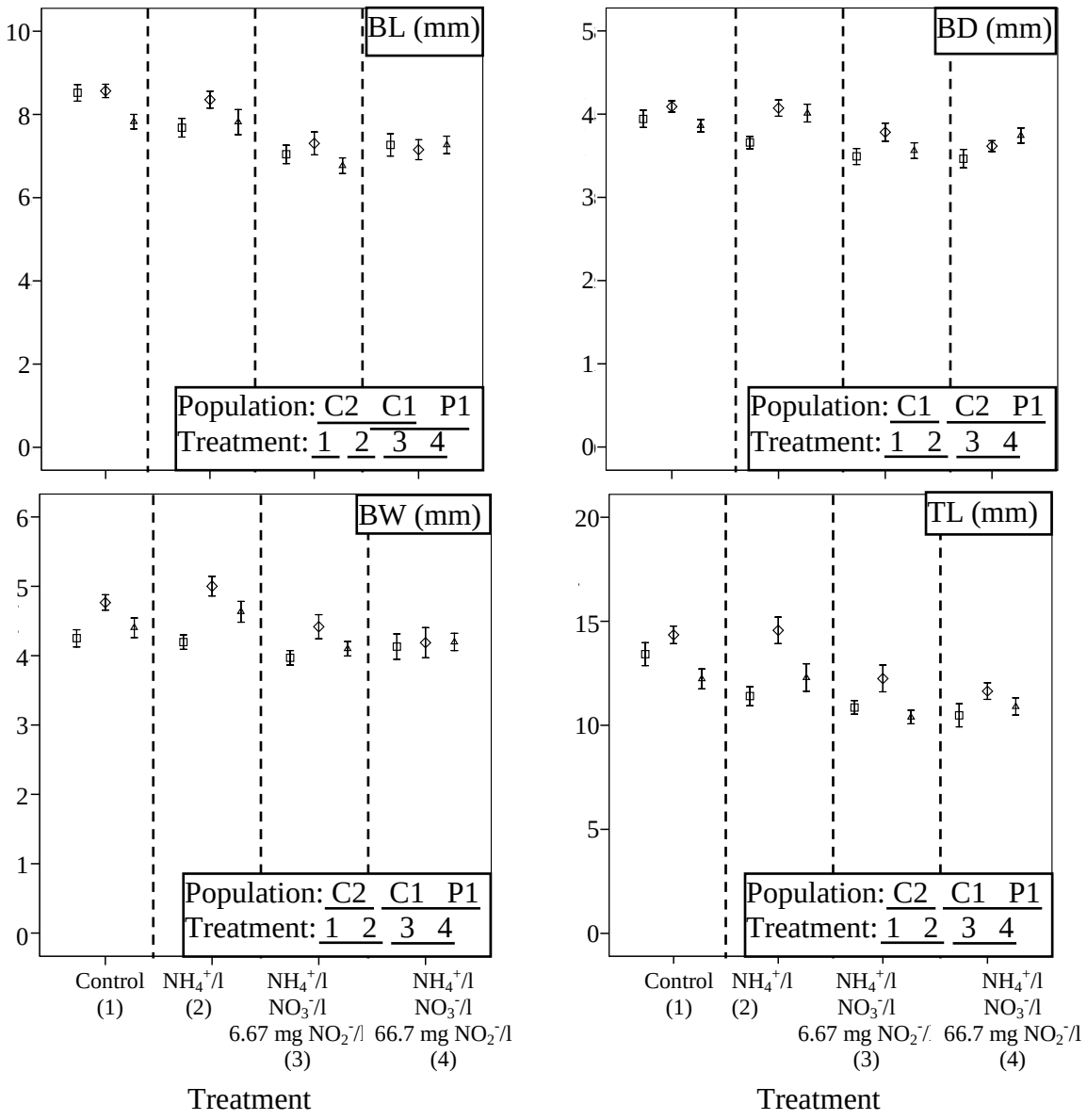

Fig. 6. Morphological traits (mean ±1 SE) of *Pelophylax perezi* tadpoles exposed to different nitrogenous treatments in the common garden experiment. Results of HDS Tukey´s test (α = 5%) for treatment are shown (common underbars notation). Treatment codes are shown in brackets. □ C1; ◇ C2; △ P1.

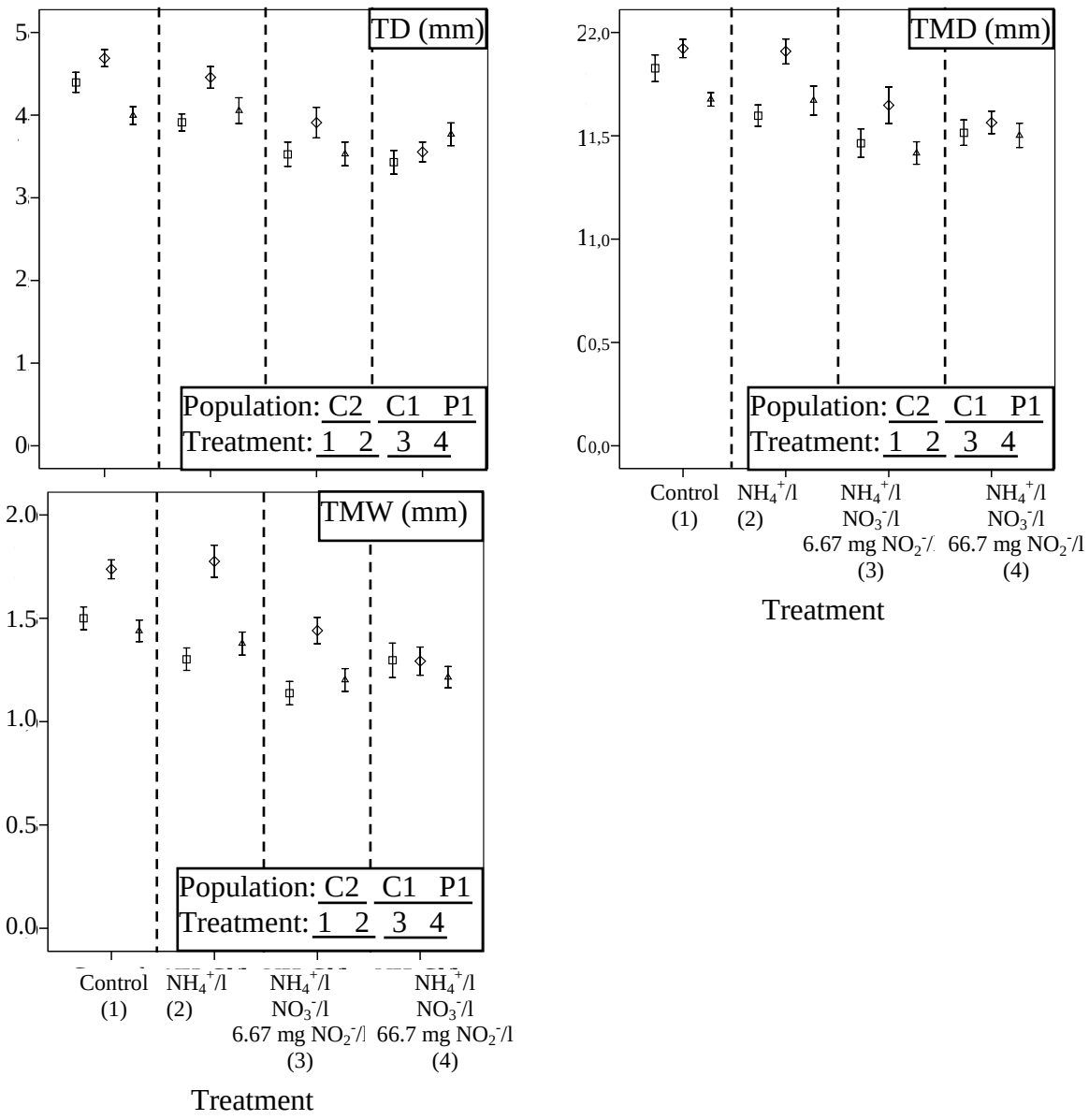

Fig. 6 (continued). Morphological traits (mean ±1 SE) of *Pelophylax perezi* tadpoles exposed to different nitrogenous treatments in the common garden experiment. Results of HDS Tukey´s test (α = 5%) for treatment are shown (common underbars notation). Treatment codes are shown in brackets. □ C1; ◇ C2; △ P1.

Larval final mass and growth

Final mass and growth sources of variation appears in Table 5. The exposure to mixtures of nitrogenous compounds reduced their mean value for all studied populations (Fig. 7). A significant effect of population of origin on final mass was found when initial mass was taken into account (Table 5), being larvae from C2 larger than those from the other populations. The interaction population of origin x treatment only was significant for final mass (Table 5). Larvae from population C1 and C2 reduced mass when they were exposed to treatment 2 (40 mg NH_4Cl/l), whereas the polluted population (P1) kept constant its mass at this treatment.

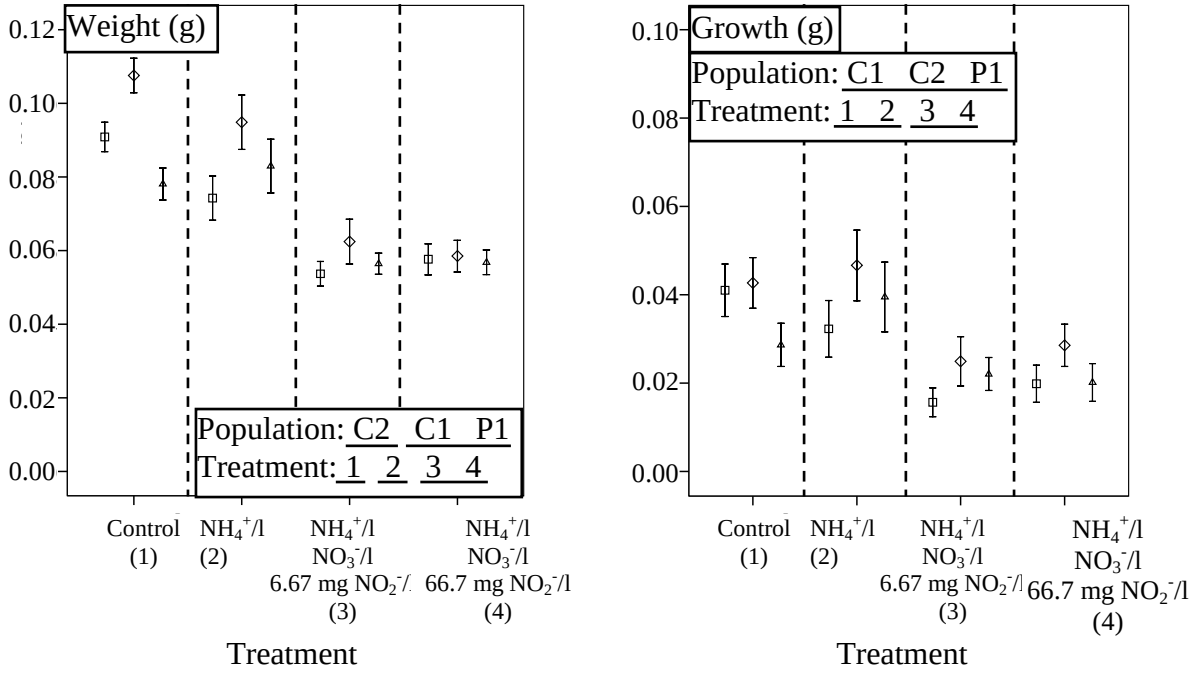

Fig. 7. Final weight and growth (g) (mean ± 1 SE) of larvae of *Pelophylax perezi* exposed to different nitrogenous treatments in the common garden experiment. Results of HDS Tukey´s test (α = 5%) for treatment are shown (common underbars notation). Treatment codes are shown in brackets. □ C1; ◇ C2; △ P1.

Table 5. Summary statistics for ANCOVAs performed on larvae mass and growth after 21 days of exposure in mesoscom conditions. All variables were log-transformed. ndf: numerator degrees of freedom; ddf: denominator degrees of freedom. NA: not applicable.

	Not correcting for initial mass				Correcting for initial mass			
Source of variation	ndf	ddf	F	*P*	ndf	ddf	F	*P*
Final mass								
Initial mass	NA	NA	NA	NA	1	195	0.423	0.516
Population of origin	2	196	10.487	0.0001	2	195	9.964	0.0001
Treatment	3	12.006	4.179	0.031	3	12.028	4.165	0.031
Pool(Treatment)	12	196	18.725	0.0001	12	195	18.510	0.0001
Population x Treatment	6	196	2.460	0.026	6	195	2.487	0.024
Growth								
Initial mass	NA	NA	NA	NA	1	195	75.484	0.0001
Population of origin	2	196	3.115	0.047	2	195	1.789	0.170
Treatment	3	12.022	1.994	0.169	3	12.079	3.641	0.045
Pool(Treatment)	12	196	5.350	0.0001	12	195	6.438	0.0001
Population x Treatment	6	196	0.589	0.739	6	195	0.240	0.963

Field experiment

Water physicochemistry

Physicochemical characteristics of water were significantly affected by localities and time of measurement, which also affected the influence of the localities on water characteristics (Table 6). The lowest pH, conductivity and salinity levels were shown by L1 (pH) and L2 (conductivity and salinity), whereas L3 showed the highest values for all measured variables, with the exception of water depth (the lowest values for the studied localities) and pH (not differing with respect to L2) (Fig. 8). The significant time x experimental locality interaction recorded for all the physicochemical variables analysed (Table 6) indicates that differences among localities decreased during the experiment to increase again by its end, with the exception of water temperature and depth (Fig. 8).

As regards to nitrogenous ion concentration, the reduced number of cases for which detectable ammonium levels were recorded for any locality prevented its analysis. In relation to nitrite concentration, only L2 and L3 had reliable data and L3 showed higher nitrite concentration. Nitrate concentration was higher for L2 population. L1 showed the lowest value for nitrate concentration (Table 7; Fig. 9).

Table 6. Summary statistics of repeated measure ANOVA on physicochemical characteristics of water in the localities selected for the field experiment. df: degrees of freedom.

Variable	Source of variation	df	F	P
pH	Locality	2	18.691	0.0001
	Time	3	13.181	0.0001
	Time x Locality	6	11.536	0.0001
	Error	24		
Temperature	Locality	2	9.821	0.001
	Time	3	20.023	0.0001
	Time x Locality	6	7.351	0.0001
	Error	24		
Conductivity	Locality	2	987.642	0.0001
	Time	3	38.375	0.0001
	Time x Locality	6	37.351	0.0001
	Error	24		
Salinity	Locality	2	982.697	0.0001
	Time	3	43.605	0.0001
	Time x Locality	6	41.942	0.0001
	Error	24		
Depth	Locality	2	173.342	0.0001
	Time	3	3.126	0.045
	Time x Locality	6	4.420	0.004
	Error	24		

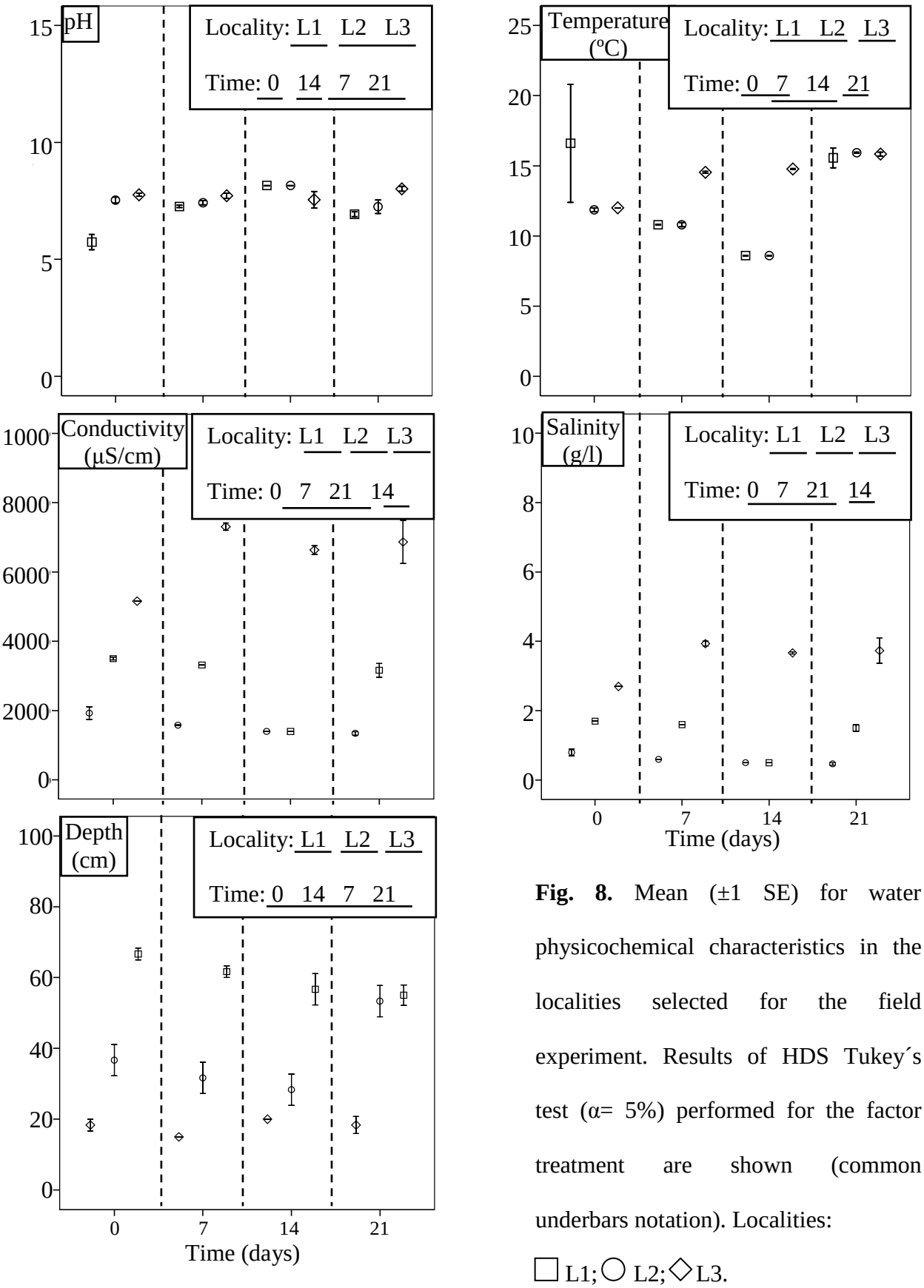

Fig. 8. Mean (±1 SE) for water physicochemical characteristics in the localities selected for the field experiment. Results of HDS Tukey´s test (α= 5%) performed for the factor treatment are shown (common underbars notation). Localities: □ L1; ○ L2; ◇ L3.

Table 7. Summary statistics of ANOVAs on nitrogenous ion concentration in the localities selected for the field experiment. NA: not detectable level of NH_4^+ in any location; *: not detectable NO_2^- in L1 locality only L2 and L3 localities could be compared. ndf: numerator degrees of freedom; ddf: denominator degrees of freedom.

Variable	Source of variation	nddf	ddf	F	P
Ammonium	Locality	NA	NA	NA	NA
Nitrite	Locality	1*	4	9.009	0.040
Nitrate	Locality	2	8	254.824	0.0001

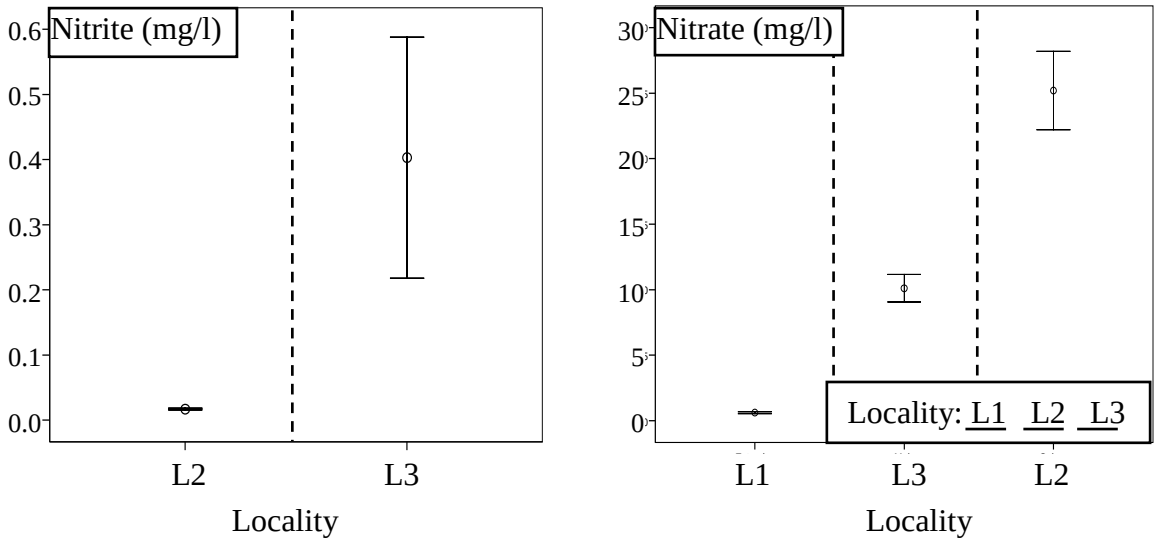

Fig. 9. Mean (±1 SE) for nitrite and nitrate concentrations (mg/l) in the localities selected for the field experiment. Results of HDS Tukey´s test (α = 5%) for treatment are shown (common underbars notation).

Larval mortality

Mean larval mortality was significantly only affected by the experimental locality (Table 8). The exposure to the localities L2 and L3 produced higher mortality than in the case of L1 (Fig. 10).

Larval morphology

Larvae exposed to L3 locality showed a trend to have higher values for absolute morphology (Fig. 11). Such influence is generally also detected, at least marginally, when analyses were performed correcting for initial mass, but not when the correction was made for final mass (Table 9). Larvae from C1 population showed higher mean values for BL, TL, TMD and TMW, although HDS Tukey´s test showed significant differences among populations only for BL, TMD and TMW (Table 9; Fig. 11). No significant population of origin x locality interactions were detected in any case.

Table 8. Summary statistics of GLZ for binary data for larval mortality after 21 days of exposure in enclosures in the field. df: degrees of freedom. NA: not applicable.

	Not correcting for initial mass				Correcting for initial mass			
Source of variation	df	Log-likelihood	X^2	P	df	Log-likelihood	X^2	P
Population of origin	2	-32.662	2.678	0.262	2	-32.662	2.678	0.262
Locality	2	-29.491	6.342	0.0420	2	-29.491	6.342	0.0420
Enclosure(Locality)	6	-25.732	7.517	0.276	6	-25.732	7.517	0.276
Population x Locality	4	-24.016	3.433	0.488	4	-24.016	3.433	0.488
Initial mass	NA	NA	NA	NA	1	-23.211	1.609	0.205

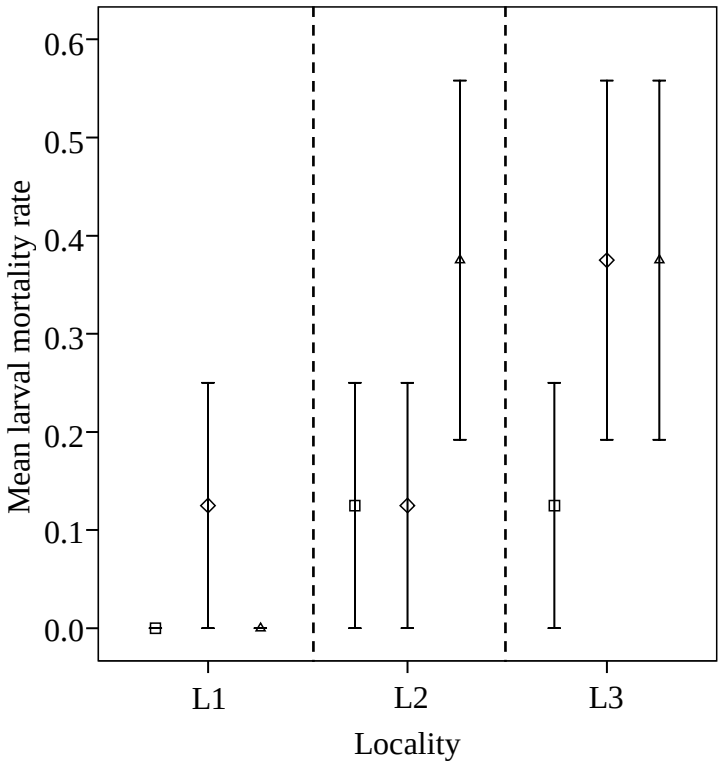

Fig. 10. Mean (± 1 SE) mortality of larvae of *Pelophylax perezi* exposed to different field localities for 21 days in the field experiment. Source populations: □ C1; ◇ C2; △ P1.

Table 9. Summary statistics of ANCOVAs performed on the morphological traits of tadpoles after 21 of exposure in the field experiment. All variables were log-transformed. * P<1%; ** P<5%; ***P<10%. NA: not applicable.

Variable	Source of variation	Not correcting for mass	Correcting for initial mass	Correcting for final mass
BL	Population	$F_{2, 44}$= 4.207**	$F_{2, 43}$= 1.418	$F_{2, 43}$= 2.867***
	Locality	$F_{2, 5.913}$= 5.759**	$F_{2, 7.427}$= 3.446***	$F_{2, 8.648}$= 0.317
	Mass	NA	$F_{1, 43}$= 8.016*	$F_{1, 43}$= 34.428*
	Enclosure(Locality)	$F_{6, 44}$= 1.545	$F_{6, 43}$= 1.026	$F_{6, 43}$= 0.950
	Population x Locality	$F_{4, 44}$= 0.526	$F_{4, 43}$= 0.392	$F_{4, 43}$= 0.459
BD	Population	$F_{2, 44}$= 1.173	$F_{2, 43}$= 0.409	$F_{2, 43}$= 0.235
	Locality	$F_{2, 5.907}$= 4.979***	$F_{2, 7.312}$= 3.475***	$F_{2, 7.635}$= 0.135
	Mass	NA	$F_{1, 43}$= 2.859***	$F_{1, 43}$= 25.126*
	Enclosure(Locality)	$F_{6, 44}$= 1.453	$F_{6, 43}$= 1.113	$F_{6, 43}$= 1.498
	Population x Locality	$F_{4, 44}$= 1.264	$F_{4, 43}$= 0.606	$F_{4, 43}$= 1.028
BW	Population	$F_{2, 44}$= 1.234	$F_{2, 43}$= 0.063	$F_{2, 43}$= 0.750
	Locality	$F_{2, 5.894}$= 11.966*	$F_{2, 7.401}$= 10.180*	$F_{2, 9.443}$= 4.657**
	Mass	NA	$F_{1, 43}$= 6.329**	$F_{1, 43}$= 27.736*
	Enclosure(Locality)	$F_{6, 44}$= 1.274	$F_{6, 43}$= 1.045	$F_{6, 43}$= 0.744
	Population x Locality	$F_{4, 44}$= 2.330***	$F_{4, 43}$= 0.673	$F_{4, 43}$= 1.077
TL	Population	$F_{2, 44}$= 2.905***	$F_{2, 43}$= 0.588	$F_{2, 43}$= 3.300**
	Locality	$F_{2, 5.893}$= 6.741**	$F_{2, 7.866}$= 5.474**	$F_{2, 10.979}$= 0.639
	Mass	NA	$F_{1, 43}$= 4.205**	$F_{1, 43}$= 26.544*
	Enclosure(Locality)	$F_{6, 44}$= 1.263	$F_{6, 43}$= 0.795	$F_{6, 43}$= 0.531
	Population x Locality	$F_{4, 44}$= 1.866	$F_{4, 43}$= 0.660	$F_{4, 43}$= 0.888

Table 9 (continued). Summary statistics of ANCOVAs performed on the morphological traits of tadpoles after 21 of exposure in the field experiment. All variables were log-transformed. * P<1%; ** P<5%; ***P<10%. NA: not applicable.

Variable	Source of variation	Not correcting for mass	Correcting for initial mass	Correcting for final mass
TD	Population	$F_{2, 44}$= 2.122	$F_{2, 43}$= 0.156	$F_{2, 43}$= 2.091
	Locality	$F_{2, 5.857}$= 8.111**	$F_{2, 7.579}$= 2.497	$F_{2, 8.191}$= 1.061
	Mass	NA	$F_{1, 43}$= 6.113**	$F_{1, 43}$= 35.211*
	Enclosure(Locality)	$F_{6, 44}$= 0.941	$F_{6, 43}$= 0.932	$F_{6, 43}$= 1.135
	Population x Locality	$F_{4, 44}$= 1.124	$F_{4, 43}$= 0.212	$F_{4, 43}$= 0.528
TMD	Population	$F_{2, 44}$= 4.647**	$F_{2, 43}$= 1.353	$F_{2, 43}$= 4.126**
	Locality	$F_{2, 5.803}$= 6.474**	$F_{2, 10.588}$= 3.649***	$F_{2, 17.682}$= 0.266
	Mass	NA	$F_{1, 43}$= 4.366**	$F_{1, 43}$= 26.190*
	Enclosure(Locality)	$F_{6, 44}$= 0.684	$F_{6, 43}$= 0.344	$F_{6, 43}$= 0.245
	Population x Locality	$F_{4, 44}$= 1.397	$F_{4, 43}$= 0.311	$F_{4, 43}$= 0.564
TMW	Population	$F_{2, 44}$= 5.775*	$F_{2, 43}$= 2.487***	$F_{2, 43}$= 4.108**
	Locality	$F_{2, 5.845}$= 11.046**	$F_{2, 9.524}$= 9.942*	$F_{2, 14.204}$= 1.959
	Mass	NA	$F_{1, 43}$= 7.090**	$F_{1, 43}$= 20.044*
	Enclosure(Locality)	$F_{6, 44}$= 0.872	$F_{6, 43}$= 0.438	$F_{6, 43}$= 0.338
	Population x Locality	$F_{4, 44}$= 3.557**	$F_{4, 43}$= 0.869	$F_{4, 43}$= 1.960

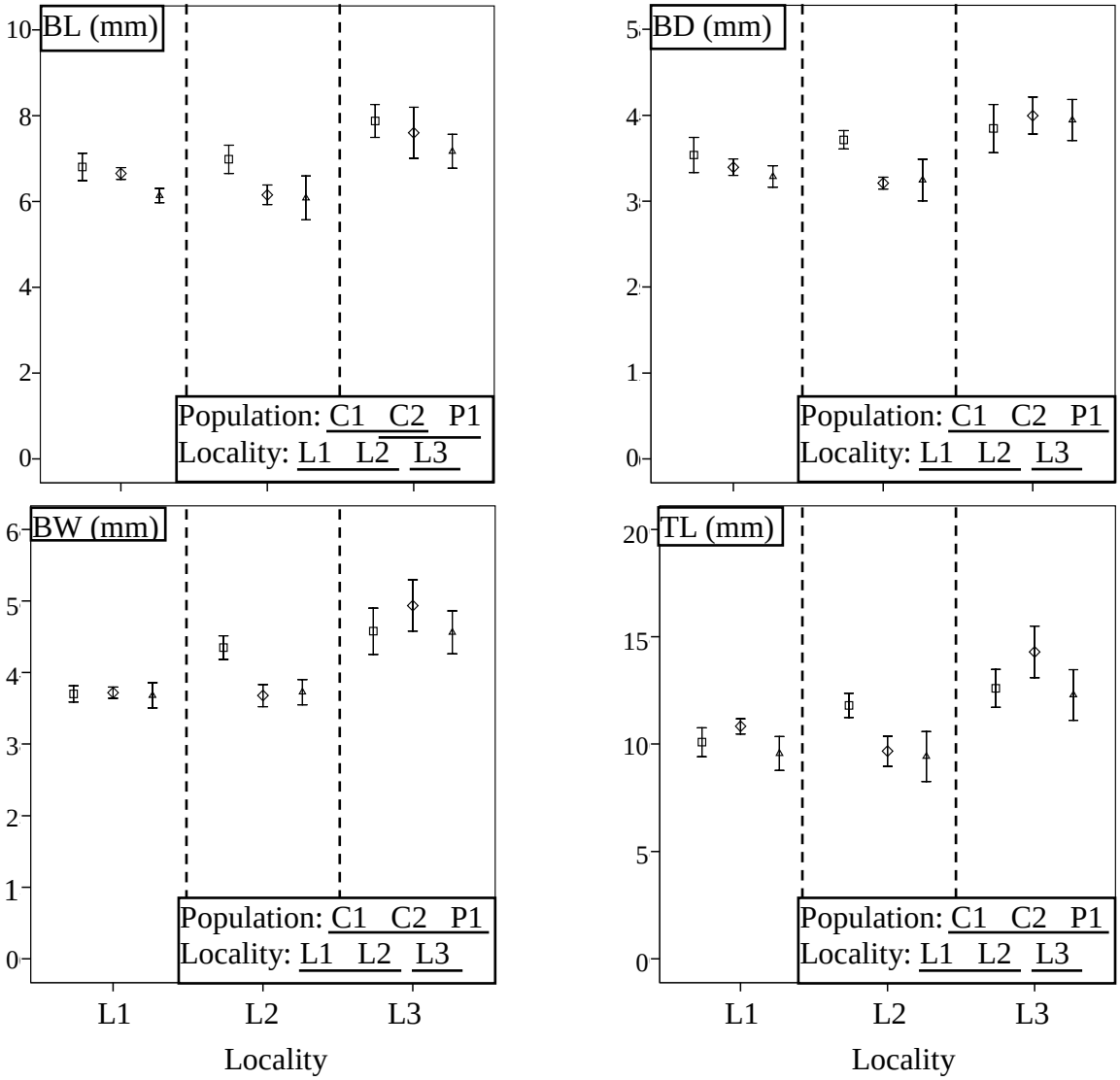

Fig. 11. Morphological traits (mean ±1 SE) of *Pelophylax perezi* tadpoles raised at different field localities for 21 days in the field experiment. Results of HDS Tukey´s test (α = 5%) for treatment are shown (common underbars notation). Source populations: □ C1; ◇ C2; △ P1.

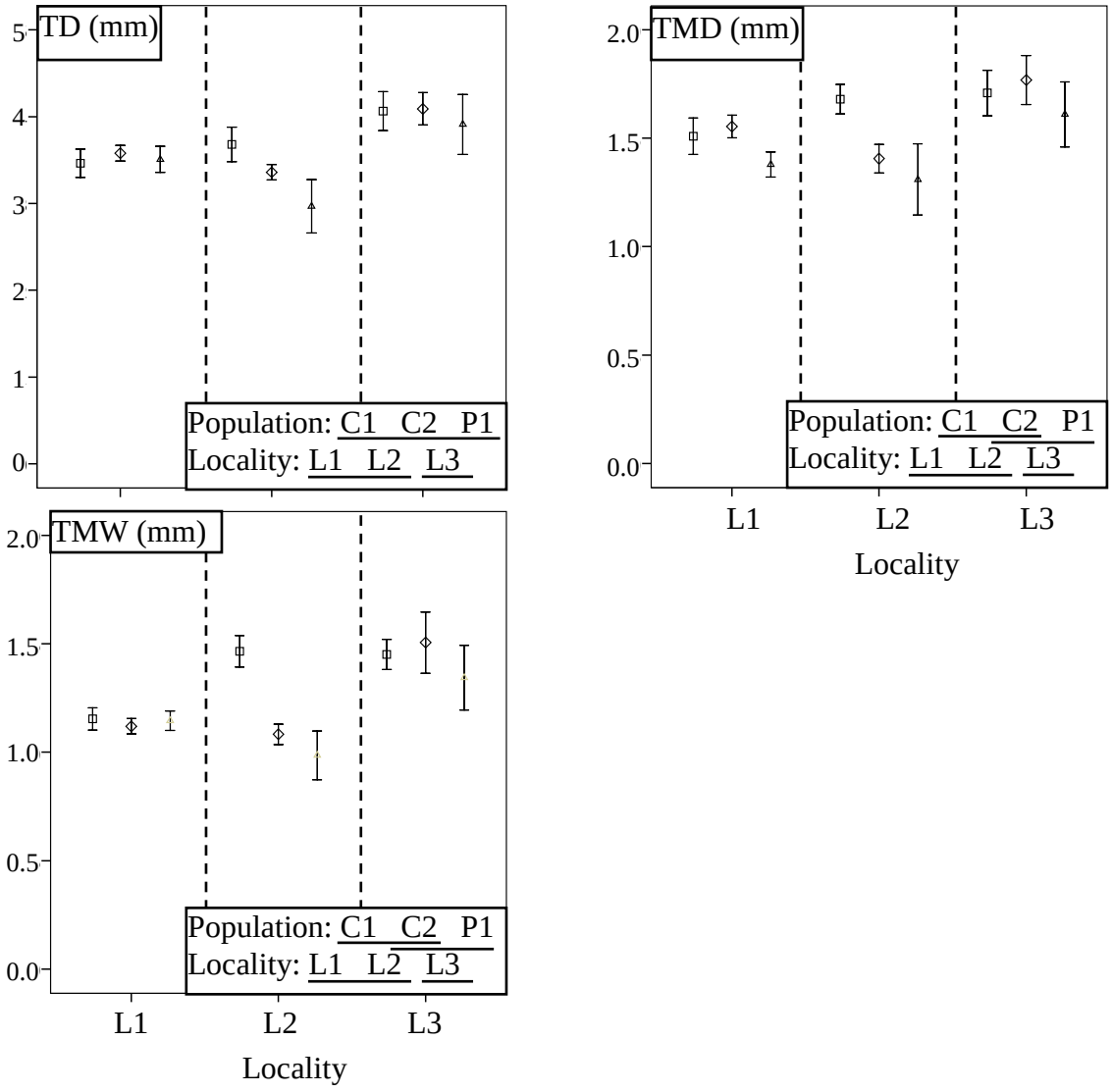

Fig. 11 (continued). Morphological traits (mean ±1 SE) of *Pelophylax perezi* tadpoles raised at different field localities for 21 days in the field experiment. Results of HDS Tukey´s test (α = 5%) for treatment are shown (common underbars notation). Source populations: □ C1; ◇ C2; △ P1.

Larval final mass and growth

Final mass and growth were significantly affected by locality (Table 10). Those larvae exposed to L3 showed higher mean values for these variables than those exposed to the rest of localities (Fig. 12).

Table 10. Summary statistics for ANCOVAS performed on larvae mass and on larval growth after 21 days of exposure in enclosures in the field. All variables were log-transformed. ndf: numerator degrees of freedom; ddf: denominator degrees of freedom. NA: not applicable.

	Not correcting for initial mass				Correcting for initial mass			
Source of variation	ndf	ddf	F	*P*	ndf	ddf	F	*P*
			Final mass					
Initial mass	NA	NA	NA	NA	1	43	11,871	0.001
Population of origin	2	44	1.622	0.209	2	43	1,425	0.252
Locality	2	5.851	16.090	0.004	2	10.701	17,900	0.0001
Enclosure(Locality)	6	44	0.902	0.502	6	43	0,336	0.914
Population x Locality	4	44	1.598	0.192	4	43	0,123	0.974
			Growth					
Initial mass	NA	NA	NA	NA	1	43	0.105	0.747
Population of origin	2	44	1.863	0.167	2	43	1.876	0.165
Locality	2	5.395	34.330	0.001	2	13.477	18.384	0.0001
Enclosure(Locality)	6	44	0.219	0.969	6	43	0.221	0.968
Population x Locality	4	44	0.168	0.954	4	43	0.079	0.988

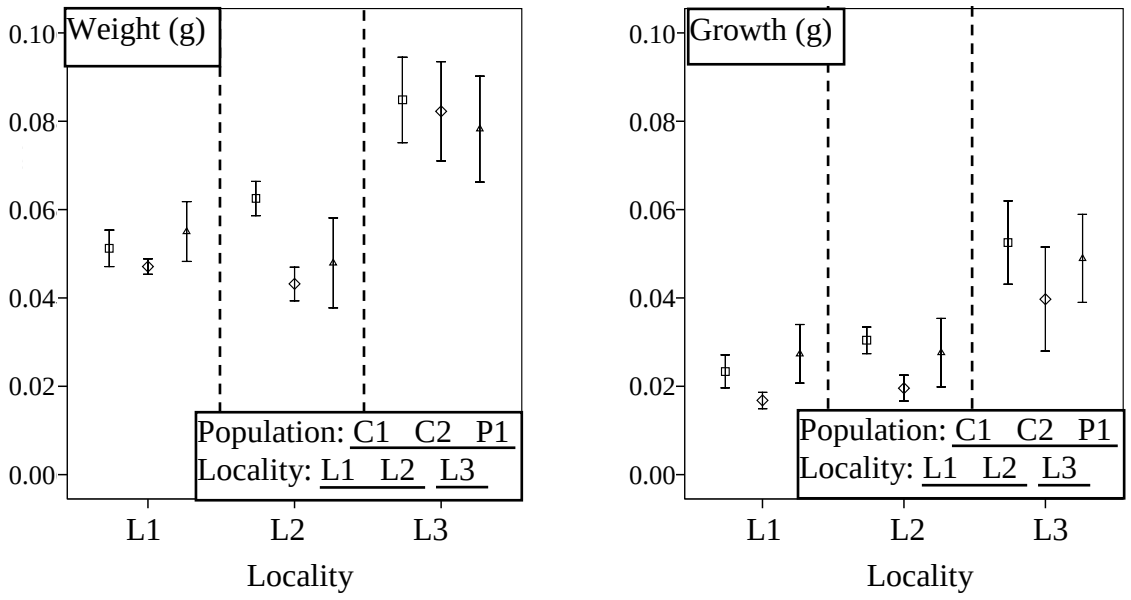

Fig. 12. Final weight (a) and growth (b) (mean ± 1 SE) of larvae of *Pelophylax perezi* raised at different field localities for 21 days in the field experiment. Results of HDS Tukey´s test (α = 5%) for treatment are shown (common underbars notation). Source populations: □ C1; ◇ C2; △ P1.

DISCUSSION

The results obtained suggest that the exposure to stressing aquatic environments significantly affected *P. perezi* larval survival, final size, growth and morphology. Nevertheless, since the direction of the influence differed between the common garden and the field experiment, it would be necessary to integrate both approaches to accurately determine the impact of nitrogenous pollution on the study species and to provide generality to our results.

Some previous studies have described that the exposure to nitrogenous treatments in outdoor mesocosms increased larval mortality (de Wijer *et al.*, 2003). However, other evidences found no effects in such conditions (Hatch & blaustein, 2003; Boone *et al.*,

2005; Boone *et al.,* 2007). Our results agree with this latter scenario. The observed tolerance *P. perezi* may be a consequence of either any highly effective detoxification pathway or it may be mediated by enhanced algal growth at polluted conditions. Ammonium, nitrite and nitrate concentrations corresponding to polluted treatments may explain the detected periphyton biomass (Camargo & Alonso, 2006), which would have contributed to reduce the concentrations of the nitrogenous ions to that experimental individuals were actually exposed. In laboratory conditions, Egea-Serrano *et al.* (2009) reported high larval mortality for *P. perezi* for the same nominal concentrations considered in the present study. Although the exposure to stressing factors, such as salinity, in mesocosm conditions was more stressful than in the laboratory in *Bufo calamita* larvae (Gómez-Mestre & Tejedo, 2003), the observed divergence found between experimental conditions may rely both on the controlled conditions in the laboratory, and the high rate of treatment renewal, that would have contributed to keep stable the nominal concentrations selected, representing a more severe stress to experimental individuals when compared with more natural conditions. This hypothesis emphasizes the difficulty of generalizing the conclusions obtained in the laboratory to understand the response of natural amphibian populations to stressing factors (Boone & James, 2005). However, results obtained for the most natural approach (i.e. field experiment) indicates that larval *P. perezi* may be threatened by nitrogenous pollution, which agrees with the increased mortality reported for enclosured larvae of different amphibian species in manipulative field experiments and field assays (Griffis-Kyle & Ritchie, 2007; Peltzer *et al.,* 2008; but see de Wijer *et al.,* 2003 for survival at hatching).

Nevertheless, the higher larval mortality reported for the polluted sites cannot be totally attributable to nitrogenous pollution, as higher nitrogenous ion concentrations (i.e. those used for the common garden experiment) did not increase mortality.

Moreover, it is unlikely that this result can be completely attributed to any hypothetic osmotic response, since the trends for larval mortality and water salinity in relation to experimental localities are different. Thus, additional stressing factors could be involved in the lethal effects observed, although no further explanations may be proposed considering the available data.

Experiments performed in mesocom conditions have revealed either the absence of significant effects (Boone *et al.*¸ 2005) or the existence of positive influence of nitrogenous treatments on amphibian larvae growth (de Wijer *et al.*, 2003; Hatch & Blaustein, 2003; Boone *et al.*¸ 2007). Nevertheless, our results disagree with the existing information, since, although the effects produced by ammonium acting isolately did not differ with respect to control, treatments corresponding to the combinations of nitrogenous compounds reduced both final larval weight and growth. Because the fact that periphyton biomass was greater at the polluted treatments, we could expect a facilitation of beneficial effects on larvae growth due to an increase in food availability with respect to control, as it has been previously suggested in other settings (Boone *et al.*, 2005; 2007). However, Egea-Serrano *et al*. (2009) reported for laboratory experiments a lower feeding efficiency for *P. perezi* larvae at polluted treatments but growth was likewise depressed. So, the exposure to the nitrogenous compound mixtures, although representing an enriched environment, results harmful to *P. perezi* both at the laboratory and mesocosm conditions. Contrarily to laboratory and mesocosms, larvae exposed to natural conditions at the field experiment increased both final weight and growth at polluted localities. This result can be due to an indirect effect of pollution via food web (de Wijer *et al.*¸ 2003). Although periphyton biomass could not be measured properly, it is reasonable to assume that polluted sites would show high algal growth, since nutrient concentration was significantly higher than control site (see

Camargo & Alonso, 2006). So, the higher mass recorded for surviving larvae exposed to polluted localities could be explained by considering that these larvae could be able to perform an overcompensation response to the stress produced by pollutants by increasing their feeding efficiency.

The exposure to stressing factors, such as dessication, predator and competitor presence, low temperature, starvation or osmotic stress has been shown to affect both larval and juvenile morphology in anurans (Relyea, 2002a, b; Gómez-Mestre & Tejedo, 2005; Merilä *et al.*¸2004; Richter-Boix *et al.*, 2006). No much information exist on the effect of nitrogenous pollution on morphological variation in larval amphibians, with the exception of body length in laboratory experiments (e.g. Oromí *et al.*, 2009). Polluted treatments in the mesocoms and localities at the field experiment either reduced or increased, respectively, absolute values of morphological traits (at least marginally). This disagreement could be explained by considering that the stress produced by the exposure to polluted localities was high enough to induce some overcompensation response in the tadpoles, whereas polluted treatments in the mesocosms were so harmful than tadpoles could not perform such response.

Since larval growth and morphology has been related to swimming performance (e.g. Watkins, 1997; Van Buskirk & McCollum, 2000; Arendt, 2003; Dayton *et al.*, 2005), the results obtained suggest that nitrogenous pollution may affect tadpole competitive abilities and the risk of being predated (Kupferber, 1998; Dayton & Fitzgerald, 2001). Relyea (2002b) suggested that the development of plastic phenotypes in presence of predators was an adaptive response, that does not imply evolutionary change in mean trait (Gotthard & Nylin, 1995).

Larval mortality was not increased at the polluted treatments for the common garden experiment, but it was augmented at localities showing the highest nitrite and

nitrate concentrations (L2 and L3 localities). Therefore, it is difficult to infer the positive effects that pollution-induced morphologies may represent, as the adaptive hypothesis would suggest (Gotthard & Nylin, 1995). Moreover, mean mortality for each mesocosm (n = 16) or enclosure (n = 9) was not correlated with mean larval mass morphology (Pearson correlation, $P > 0.05$ in all cases). Further studies are needed to fully understand the implications of the morphological responses to pollution described.

Significant population x treatment interactions have been found for some morphological variables as well as for larval final mass in the common garden experiment. This result suggests the existence of population-specific differences and possible local adaptation in the tolerance to nitrogenous pollution, as it has been previously described for laboratory assays (Egea-Serrano *et al.*, 2009). This fact would agree with the intraspecific variation in the tolerance to fertilizers described for different amphibian species in previous studies (Hecnar, 1995; Johansson *et al.*, 2001; Hatch & Blaustein, 2003; Macías *et al.*, 2007; Shinn *et al.*, 2008). However, the results obtained for the field experiment did not fit with a local adaptation hypothesis since populations did not differ in their response to the destination localities. This could be attributed to the fact that natural environments were not stressful enough to magnify the different responses of larvae from each population to pollution.

In conclusion, the results showed that in the natural environments, larval *P. perezi* are tolerant, at least to some extent, to nitrogenous pollution. Nevertheless, the presence of different nitrogenous compounds acting in combination and hypothetically acting with additional stressing factors would make larvae vulnerable to pollution, since mortality, as well as morphology and size were affected. Morphology may affect performance abilities (Van Buskirk & McCollum, 2000), decreasing escape abilities which may increase the risk of being predated by gape-limited predators (Wellborn,

1996). The reduction in larval size may determine smaller size at metamorphosis that indirectly may retard the age and to sexual maturation and decrease adult size (Smith, 1987). Therefore, the non-lethal indirect effects of pollution may have deleterous effects on population growth of *P. perezi*. Nevertheless, further studies are needed to assess whether the effects of nitrogenous pollution detected for larval developmental stages ensue to the terrestrial phase of amphibian life cycle.

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CAPÍTULO 8

ANALYSIS OF THE EFFECTS OF NITROGENOUS POLLUTION ON SWIMMING PERFORMANCE OF LARVAE OF *PELOPHYLAX PEREZI* (SEOANE, 1885) THROUGH ASSAYS REPRESENTATIVE OF NATURAL CONDITIONS

Abstract.- Although many studies have assessed the impact of pollutants on amphibian behavior, information regarding their effect on locomotor abilities is scarce. However, studies analyzing the impact of nitrogenous compounds on such abilities, as well as the intra-specific variation of the tolerance to pollutants, is completely lacking. Therefore, in two different experiments carried out in mesocosms and enclosures, respectively, we examined the effect of the exposure to NH_4Cl (isolated or combined with $NaNO_2$ and $NaNO_3$) and to natural streams differing in their degree of pollution on larval speed and distance swum by larvae of *Pelophylax perezi* from three populations exposed to different degrees of eutrophication. The results suggest that the exposure to nitrogenous compounds in both mesocosm and natural conditions has no direct effect on larval swimming performance, although it may have deleterous effects on the viability of larvae by affecting the relationships between morphology or growth and the swimming abilities.

Key words: Nitrogenous pollution, inter-populational variation, *Pelophylax perezi*, swimming performance, larvae, natural conditions

INTRODUCTION

Amphibian larvae fitness is dependent on locomotor abilities since such capabilities may affect competitive ability (Dayton & Fitzgerald, 2001) as well as vulnerability to predators (Watkins, 1996; Kaplan & Phillips, 2006). Moreover, the effect of morphological traits on fitness may be mediated by the impact of such traits on one or more performance variables (Arnold, 1983). Thus, because of the potential ecological relevance of larval speed, burst speed is commonly considered as an ecological measure of performance and an effective method to collect information on larval fitness (Huey & Stevenson, 1979).

Information reporting the effects of pollutants such pesticides and nitrogenous compounds on larval amphibian activity has been published previously (e.g. Hecnar, 1995; Bridges, 1997, 1999; Xu & Oldham, 1997; Hatch & Blaustein, 2000; Shinn *et al.*, 2008). A regards swimming performance, (i.e. swimming speed or total distance swum), the published studies describing the effects of pollution indicate that tadpole swimming abilities generally disminished as a consequence of the exposure to either high aluminum concentration and low pH or to pesticides (Jung & Jagoe, 1995; Bridges, 1997; but see Widder & Bidwell, 2006, 2008). Nevertheless, no information assessing the impact of nitrogenous compounds on swimming performance of larval amphibians has been reported yet.

Nitrogenous compounds significantly affect lethal and sublethal parameters in larval amphibians, both in the laboratory and in more natural settings (e.g. Boone *et al.*, 2007; Peltzer *et al.*, 2008; Egea-Serrano *et al.*¸ 2009). Moreover, the exposure to nitrogenous compounds acting in combination with other stressing factors (as expected for natural settings), such as other nitrogenous substances (Egea-Serrano *et al.*¸ 2009),

UV-B radiation (Hatch & Blaustein, 2000, 2003; Macías *et al.*, 2007), low pH (Hatch & Blaustein, 2000) or pesticides (Boone *et al.*¸ 2005), may exacerbate their effects through additive or synergistic responses (Berenbaum, 1989). Additionally, nitrogenous pollution has been described to affect larval morphology (Egea-Serrano *et al.*, chapter 7 in the present thesis), which may affect tadpole locomotor ability, since evidence has accumulated that body size, growth and morphology affects larval swimming performance (e.g. Watkins, 1997; Van Buskirk & McCollum, 2000; Arendt, 2003; Dayton *et al.*, 2005). All these facts would make likely that swimming performance could be modified by nitrogenous pollution. Considering the widespread distribution of such pollution (Carpenter *et al.*, 1998) and that it is expected to increase in the future (Tilman *et al.*, 2001; Galloway *et al.*¸ 2003), studies performed to determine the effects of nitrogenous compounds (isolated and acting in combination with other factors) on tadpole swimming performance are needed to better understand their impact on amphibian populations.

To date, most of the studies dealing with amphibian ecotoxicology correspond to laboratory assays (Boone & James, 2005). Although data collected in these conditions are a starting point in understanding the effects of a pollutant, it is necessary to determine whether the effects recorded in the laboratory are also detected in the field (Boone & Bridges, 2003; Boone & James, 2005). Pond mesocoms and field enclosures have been described as effective tools to study the effect of pollutants in more realistic conditions (Boone & Bridges, 2003; Boone & James, 2005). Mesocosms have been defined as independent outdoor artificial systems (either aquatic or terrestrial) containing food webs and processes representative of natural environment, whereas enclosures can be described as permeable containers enclosing the study organisms within a particular environment, allowing environmental exchange among them (Boone

& James, 2005). The characteristics of these methodologies allow to study accurately population- and community level processes as well as to integrate multiple stressing factors in naturally changing environments (Boone & James, 2005).

Pelophylax perezi is a waterfrog widespreads through the Iberian Peninsula and Southern France (Llorente & Arano, 1997). It inhabits mainly permanent water bodies (Díaz-Paniagua, 1990). These habitats, as a consequence of farming practices, one of the most important nitrogen sources in the nature (e.g. Ritter & Bergstrom, 2001), may hold high concentrations of different nitrogen forms (e.g. for southeastern Iberian Peninsula: 154.6 mg NH_4^+/l; 74.4 mg NO_2^-/l; 333.0 mg NO_3^-/l, Suárez, personal communication). So, this species might be exposed to and threatened by nitrogen pollution through all its life cycle, because juveniles and adults uses water bodies as shelter from predators (Martín *et al.*, 2006), as foraging (Docampo & Vega, 1990) and as breeding habitat (Egea-Serrano *et al.*¸ 2005). The wide habitat niche breadth and distribution range of *P. perezi* makes possible the existence of interpopulational variation in relation to its tolerance to increased levels of nitrogenous pollution, as it has been shown in other studies (Johansson *et al.*, 2001; Egea-Serrano *et al.*, 2009).

The aims of the present study were: 1) to determine the effects of high concentration of ammonium chloride, isolated and combined with sodium nitrite and sodium nitrate, on larval speed and distance swum by larvae of *P. perezi* raised in mesocom condition (common garden experiment); 2) to determine the effects of the exposure in enclosures placed in natural streams differing in their level of nitrogenous pollution (field experiment) on larval speed and distance swum; 3) to analyse the impact of nitrogenous pollution on the relationship between larval morphology and swimming performance for both common garden experiment and field experiment; 4) to analyse the impact of nitrogenous pollution on the trade-off between larval growth and

swimming performance for both common garden experiment and field experiment (Arendt, 2003) and, 5) to evaluate whether there are evidences of variation among populations in their tolerance to nitrogenous pollution.

MATERIAL AND METHODS

Studied populations

Five different egg masses of *P. perezi* were collected from three populations located in the Segura River Basin the first fortnight of March 2007. This basin has been reported as one of the most arid and eutrophized of the Iberian Peninsula (Vidal-Abarca *et al.*, 1987; Ballester, 2003). The selected populations were naturally exposed to highly different levels of nitrogen pollution. Two of these populations corresponded to the permanent headwater stream Río Chícamo (38º12´N, 001º03´W; 170.3 m.a.s.l.) and to a seminatural pond located in the Sierra Espuña Regional Park (37º52´N, 001º30´W; 673.0 m.a.s.l.) (C1 and C2, hereafter). C1 showed low nutrient concentration with less than 5.1 mg $N\text{-}NO_3^-$/l (Vidal-Abarca *et al.*, 2000). Although no data about nutrient concentration is available for C2, it is unlike that amphibians were exposed to nitrogenous pollution since its location is remote from urban and farming activities. The land environment corresponds to bush on marls (C1) or to pine trees on limestone lithology (C2). In contrast to the previous populations, the third population is located in a semipermanent headwater stream, Rambla del Garruchal (37º57´N, 001º04´W; 346.0 m.a.s.l.) (herafter P1), which has been exposed, at least for the last 22 years, to nitrate concentration as high as 162.1 mg NO_3^-/l (Ballester, 2003) due to intensive farming activities and subsequent run-off in its basin. Its terrestrial environment corresponds with pine trees on heterogeneous carbonated materials but, through most of the course

of the stream, the habitat has been largely modified being dominated by intensive cattle exploitations. The geographical separation between populations ranged from 28.3 km to 54.9 km.

Experimental design and response variables

Developmental stage of embryos when they were collected ranged from 15 to 18 Gosner´s stage (Gosner, 1960), with no differences among populations (Chi-square, $P > 0.05$). In all cases, embryos were transported before hatching to the laboratory, where they were reared in 12 l glass aquaria containing dechlorinated tap water (pH = 8.39; conductivity = 985 μS/cm; 0.002 mg NO_2^-/l; 4.69 mg NO_3^-/l). When they reached Gosner´s 25 developmental stage, they were exposed to treatments.

Common common garden experiment.- A total of 16 plastic pools (430 l) were located in an outdoor facility at the Campus Universitario de Espinardo (Universidad de Murcia) one month prior to the beginning of the experiment. They were filled with 200 l of dechlorinated tap water. Once a week before the experiment began, each pool was inoculated with 0.5 l of water from a natural pond (Boone *et al.*¸ 2004). Since this pond (37º52´N, 001º34´W; 1124 m.a.s.l.) is located within a pine forest in a protected area of Southern Spain, it is unlikely that the water used to inoculate the pools was exposed to pollution. In the moment of beginning the experiment (26 March 2007), pools had a thin leaf litter that provided natural feeding sources to tadpoles and contributed to make nutrient dynamics more natural. Prior to placing the larvae in the pools, water volume in each pool was reduced to 150 l, to correct for differences in water volume due to evaporation. Additionally, when larvae were placed in the pools, each tank was equipped with a plastic tile (229 cm2) facing south to assess periphyton biomass (Relyea *et al.*, 2005). Each pond contained five larvae from the three study sites that

were individually placed in 1 l plastic beaker covered with 1 mm mesh lid within each pool. Initial mass of larvae did not differed in relation to their population of origin ($F_{2, 237} = 1.064$; P = 0.347): C1 (mean ± 1 SE): 0.043 g ± 0.002 g, n = 80; C2 (mean ± 1 SE): 0.046 g ± 0.002 g, n = 80; P1 (mean ± 1 SE): 0.041 g ± 0.002 g, n = 80.

Larvae were acclimatized in the pools two days before the beginning of the experiment. At this moment, one dog chow pellet (250-350 mg) was placed in each beaker. No additional food was provided to larvae during the experiment. Each pool was haphazardously assigned to one of the four following treatments: 1) control; 2) 13.5 mg NH_4^+/l; 3) 13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 6.67 mg NO_2^-/l; 4) 13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 66.7 mg NO_2^-/l. These treatments were selected because they produced the highest larval mortality in the laboratory (Egea-Serrano *et al.*, 2009) and concentrations were within those values naturally occurring in the field in the Segura River basin (e.g. 154.6 mg NH_4^+/l; 74.4 mg NO_2^-/l; 333.0 mg NO_3^-/l, Suárez, personal communication). To obtain the experimental concentrations, 40 g NH_4Cl/l, 70 g $NaNO_2$/l and 150 g $NaNO_3$/l stock solutions were pipetted directly into the pools. Each treatment was replicated four times. The experiment thus consisted in a split-plot design (Quinn & Keough 2002), where treatment was the main plot factor and the population of origin the subplot factor.

Field experiment.- Larvae from the three study populations were reared in four selected natural streams. Two of them, Rambla del Estrecho (37º46´N, 001º45´W; 476.7 m.a.s.l.) and Rambla Tejera (38º11´N, 002º07´W; 1197.92; 1197.9 m.a.s.l.), correspond, respectively, to permanent and semipermanent headwater streams located in forest natural environments, which makes unlikely the presence of nitrogenous pollution in the water column. During the exposure period, nitrogenous compounds levels were (mean ± SE): Rambla del Estrecho: 0.6 mg NO_3^-/l ± 0.08, n = 3; Rambla Tejera: 1.4 mg NO_3^-/l ±

0.3, n = 4). The rest of the destination sites, Río Quípar (38º02´N, 001º54´W; 710 m.a.s.l.) and Río Mula (38º03´N, 001º25´W; 190.2 m.a.s.l.) corresponds to permanent rivers exposed to high degree of eutrophication as a consequence of urban wastewaters and farming practices. During the exposure period, nitrogenous compounds levels were (mean ± SE): Río Quípar: 25.02 mg NO_3^-/l ± 3.0, n = 4; Río Mula: 10.1 mg NO_3^-/l ± 1.1, n = 4). The natural presence of *P. perezi* was confirmed in all of these streams.

Eight larvae from the three studied populations were placed at each experimental locality. Initial mass of larvae did not differed in relation to their population of origin ($F_{2,69}$= 1.936; P= 0.152): C1 (mean ± 1 SE): 0.033 g ± 0.002 g, n = 72; C2 (mean ± 1 SE): 0.030 g ± 0.003 g, n = 72; P1 (mean ± 1 SE): 0.029 g ± 0.003 g, n = 72. To preserve an estimation of interpopulational variation, each larvae was individually placed in 1.5 l plastic beakers covered with 1 mm mesh lid. Larvae from each population were haphazardously distributed among three different enclosures; each one separated at least 1 m of others. Larvae were feed with one dog chow pellet (250-350 mg) at the beginning of the experiment. No food was additionally provided during the rest of the experiment. All larvae exposed to Rambla Tejera died as a consequence, probably, of the increase of water turbidity occurred after heavy rains that took place during the realizarion of the experiment. Thus, this locality was removed from the subsequent statistical analyses. Therefore, the design for the field experiment consisted of one control site (Rambla del Estrecho, L1 hereafter) and two polluted sites (Río Quípar and Río Mula, hereafter L2 and L3, respectively).

For both common garden experiment and field experiment, mass of surviving larvae (±0.0001 g) was registered after 21 days of exposure. Since data regarding initial and final larval mass were recorded, larval growth was calculated (final mass-initial mass). Individual swimming speed and distance swum were measured by placing an

individual larva into a plastic aquarium (length: 40 cm; width: 1 cm). Water depth was roughly 2 cm, just enough to cover larvae and to limit swimming to a two-dimensional space. Larvae were allowed to settle for 60 s, when a swimming response was induced by stroking the tail of the larva with a glass rod. Swimming was videotaped (Olympus FE-200 digital compact camera) overhead until tail stopped beating and we estimated swimming speed (total length swum/time swimming, cm/s) and total length swum (cm). Additionally seven morphological traits were recorded for each individual (mm): body length (BL), width (BW) and depth (BD); muscle tail width (MTW) and depth (TD); tail length (TL) and depth (TD) (Fig. 1). To do so, digital images were obtained for each larvae and they were measured with the software Image-Pro Plus version 4.5.0.29 for Windows.

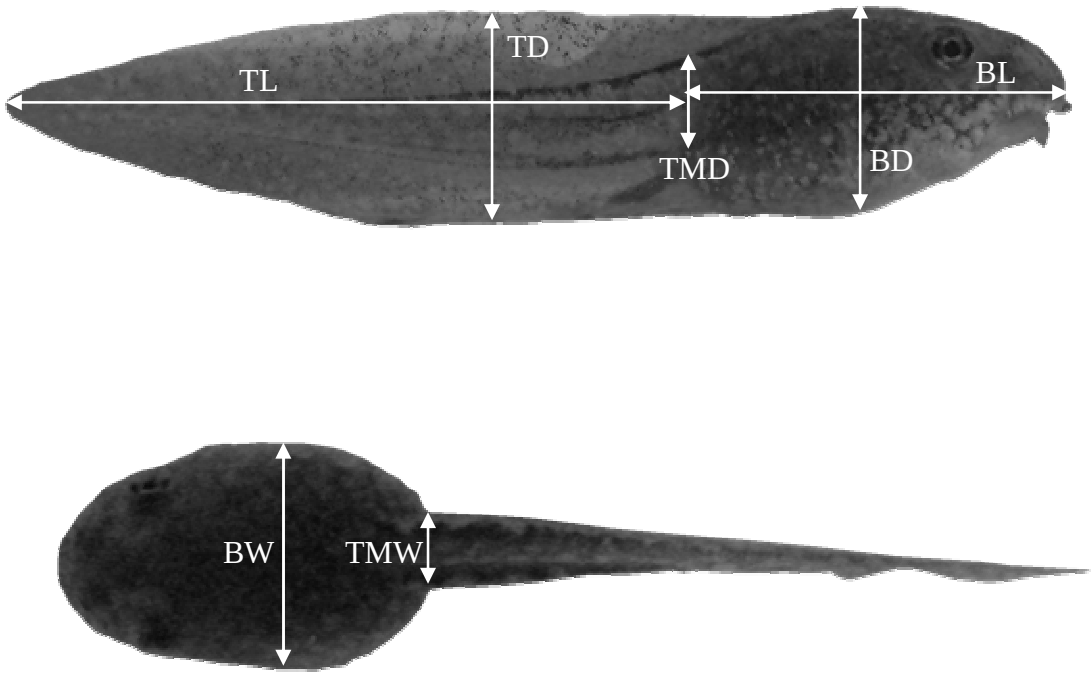


Fig. 1. Tadpole morphological measures. BL: body length; BW: body width; BD: body depth; TMW: tail muscle width; TMD: tail muscle depth; TL: tail length; TD: tail depth.

On days 2 (day 0 for the field experiment), 7, 14 and 21 after the begining of the experiment, water physicochemical characteristics (pH, temperature [ºC], conductivity [µS/cm], salinity [g/l] and depth [cm]) were measured in each pool and field localities. At the same time, a water sample was taken from each pool and field locality to establish the ammonium, nitrite and nitrate concentrations in the water column. Ammonium and nitrite analyses were performed by colorimetric methods whereas nitrate concentration was estimated by ionic chromatography. Additionally, periphyton biomass at the end of the experiment was measured for each pool to estimate a supplement resource to the tadpoles that may eventually affect their growth and perfomance (Boone *et al.*, 2005, 2007). Periphyton biomass was determined by scratching, inmediately after the experiment finished, the top surface of periphyton tiles. Once the periphyton was obtained, it was dried, allowing the waper to evaporate, and weighed (± 0.0001 g). In the case of the field experiment periphyton biomass was not enough to be measured accurately.

For the common garden and field experiments, water physicochemical variables, nitrogenous ion concentration and final periphyton biomass (only for the commong garden experiment) were analysed by ANOVAs. Treatment and time of measurement (only for physicochemical variables) were considered as fixed factor and pool as random factor nested within treatment. However, due shortage of samples with detectable values for the nitrogenous ion concentrations, both in the mesocosm and field experiments, data belonging to different tanks or enclosure were pooled, whereas time of measurement was unable to be estimated and were not considered in our design. Because periphyton biomass was estimated only at the end of the common experiment, this factor was also excluded from the statistical analysis of this variable. Additionally, since nutrient concentration estimates for the field experiment were taken outside

enclosures, the factor enclosure (nested within locality) was not considered when analysing physicochemical and nutrient data.

Larval swimming speed and distance swum, were analysed by ANCOVAs, including in the analyses population of origin, treatment (or locality) and pool or enclosure (nested within treatment or locality, respectively) as factors. Considering the hypothesis suggesting that swimming performance may be affected by both initial size (since it is correlated with egg size and, thus, of maternal induced effect [Kaplan & Phillips, 2006]) or final size, initial and final mass were included in separate analyses as covariates to correct for such influences.

Stepwise multiple regression analyses were performed to analyse the effect of morphological traits on larval swimming performance. Previous to regression analyses, homogeneity of slopes assumption was tested by ANCOVA analyses. When the influence of any morphological variables differed across population and/or treatment (or locality, for the field experiment), separate multiple regression analyses were performed for each population and treatment or locality. Since statistics for the homogeneity of slopes test could not be estimated for the field experiment, the analysis of the influence of morphology on swimming performance was not carried out in this case. The existence of a trade-off between growth and swimming variables (Arendt, 2003) was analysed using the same statistical approach than that employed for the morphology-swimming analysis.

All variables were log-transformed. All statistical analyses were performed using the statistical software SPSS® v. 15.0.

RESULTS

Common garden experiment

Water physicohemistry

Water physicochemical variables significantly differed throughout time, which also affected the effect of treatments on such parameters, with the exception of water depth and temperature (Table 1; Fig. 2). Water physicochemical characteristics (with the exception of temperature and depth) were significantly affected by treatments (Table 1). Treatments corresponding to the combination of different nitrogenous compounds showed higher values for pH than control treatment, as well as higher conductivity and salinity than the rest of treatments (Fig. 2). Furthermore, the time x treatment interaction indicates that the differences were increased with time for pH and reduced for conductivity and salinity (Table 1; Fig. 2).

Polluted treatments significantly increased ammonium, nitrite and nitrate concentrations present in the water column in relation to control treatment (Table 2; Fig. 3). Moreover, in the case of nitrite and nitrate, the combination of nitrogenous compounds increased their concentration in relation to the rest of treatments (Fig. 3).

Table 1. Summary statistics of repeated measures ANOVAs on physicochemical characteristics of water in the experimental pools for the common garden experiment. ndf: numerator degrees of freedom; ddf: denominator degrees of freedom.

Variable	Source of variation	ndf	ddf	F	P
pH	Treatment	3	12	6.325	0.008
	Pool(Treatment)	12	36	1.569	0.145
	Time	3	36	5.769	0.003
	Time x Treatment	9	36	2.442	0.028
Temperature	Treatment	3	12	1.011	0.422
	Pool(Treatment)	12	36	0.841	0.610
	Time	3	36	87.572	0.0001
	Time x Treatment	9	36	1.106	0.384
Conductivity	Treatment	3	12	54.163	0.0001
	Pool(Treatment)	23	36	1.968	0.058
	Time	3	36	180.172	0.0001
	Time x Treatment	9	36	12.072	0.0001
Salinity	Treatment	3	12	37.539	0.0001
	Pool(Treatment)	12	36	2.101	0.043
	Time	3	36	125.290	0.0001
	Time x Treatment	9	36	5.232	0.0001
Depth	Treatment	3	12	0.366	0.779
	Pool(Treatment)	12	36	7.864	0.0001
	Time	3	36	338.351	0.0001
	Time x Treatment	9	36	0.419	0.916

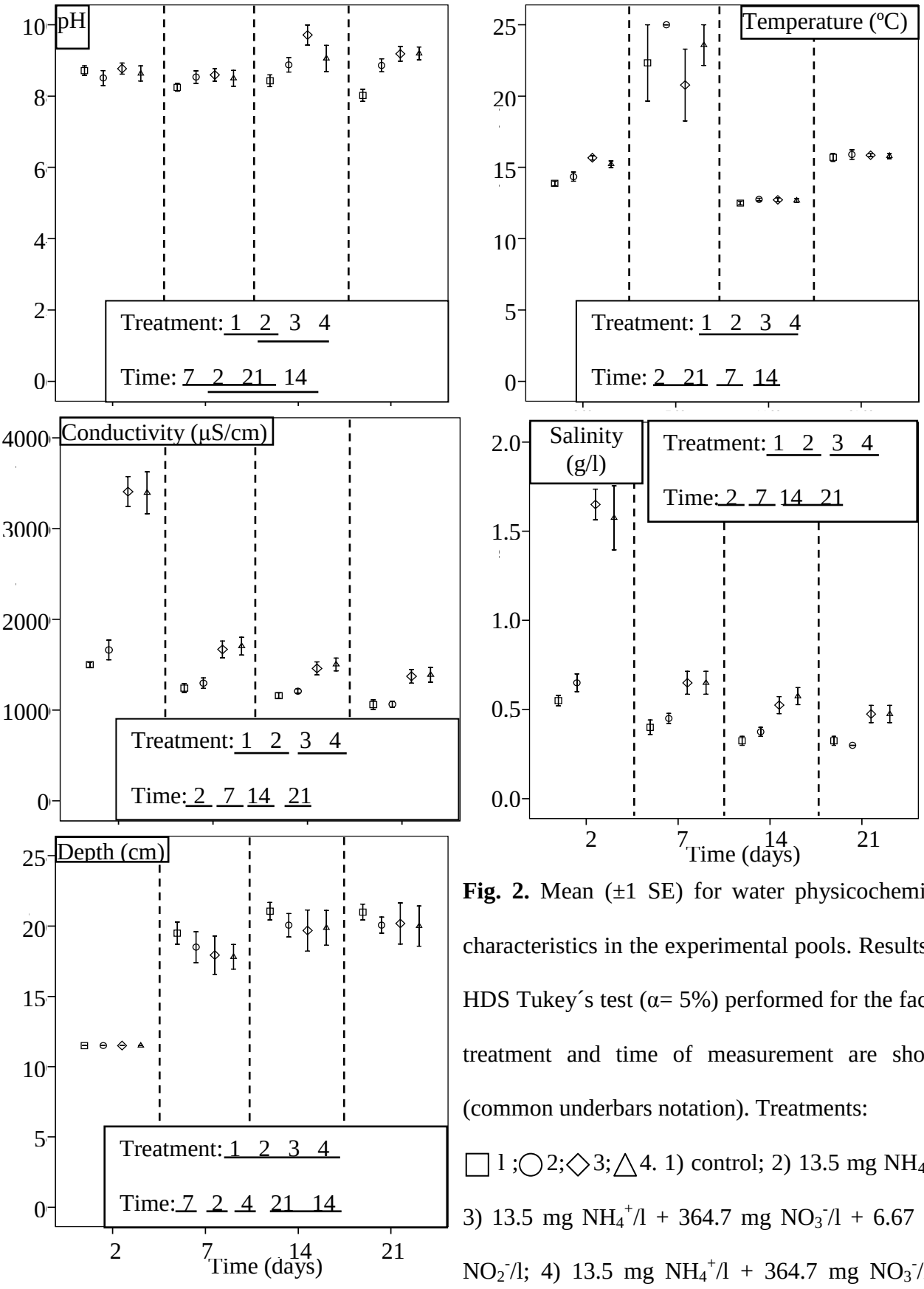


Fig. 2. Mean (±1 SE) for water physicochemical characteristics in the experimental pools. Results of HDS Tukey´s test (α= 5%) performed for the factor treatment and time of measurement are shown (common underbars notation). Treatments: □ l ;◯ 2;◇ 3;△ 4. 1) control; 2) 13.5 mg NH_4^+/l; 3) 13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 6.67 mg NO_2^-/l; 4) 13.5 mg NH_4^+/l + 364.7 mg NO_3^-/l + 66.7 mg NO_2^-/l

Table 2. Summary statistics of ANOVAs on nitrogenous ion concentration in the experimental pools for the common garden experiment. ndf: numerator degrees of freedom; ddf: denominator degrees of freedom.

Variable	Source of variation	ndf	ddf	F	P
Ammonium	Treatment	3	13.014	15.111	0.0001
	Pool(Treatment)	12	36	0.538	0.875
Nitrite	Treatment	3	11.980	19.410	0.0001
	Pool(Treatment)	11	34	3.060	0.006
Nitrate	Treatment	3	12.927	68.516	0.0001
	Pool(Treatment)	12	35	1.693	0.111

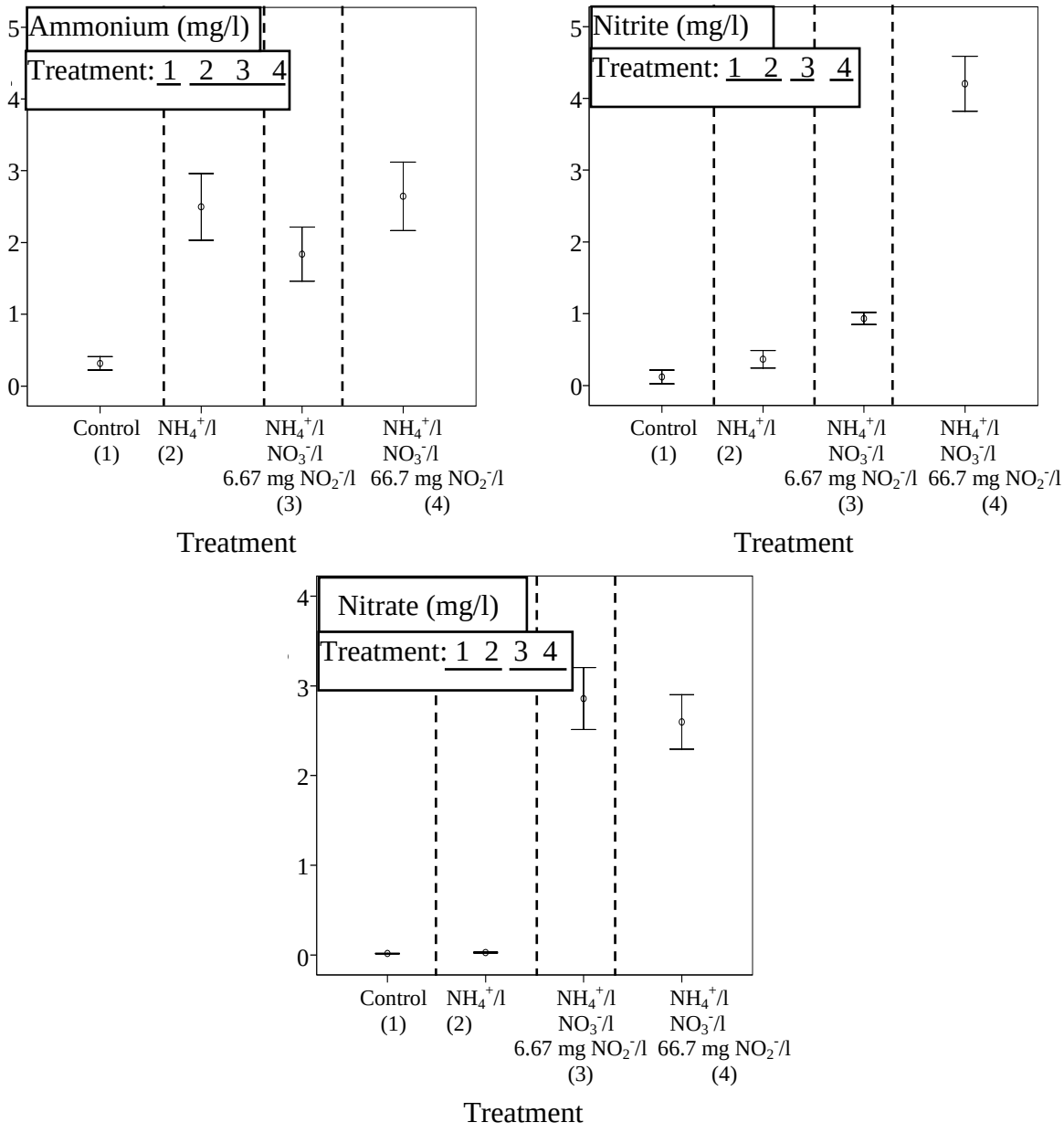


Fig. 3. Mean (±1 SE) for ammonium, nitrite and nitrate concentrations (mg/l) in the experimental pools. Treatment codes are shown in brackets. Results of HDS Tukey´s test (α = 5%) performed for the factor treatment are shown (common underbars notation).

Periphyton biomass

Growth of periphyton in the pools was significantly affected by treatments ($F_{3,12}$=23.341; P= 0.0001), being higher for those pools exposed to nitrogenous compounds (Fig. 4).

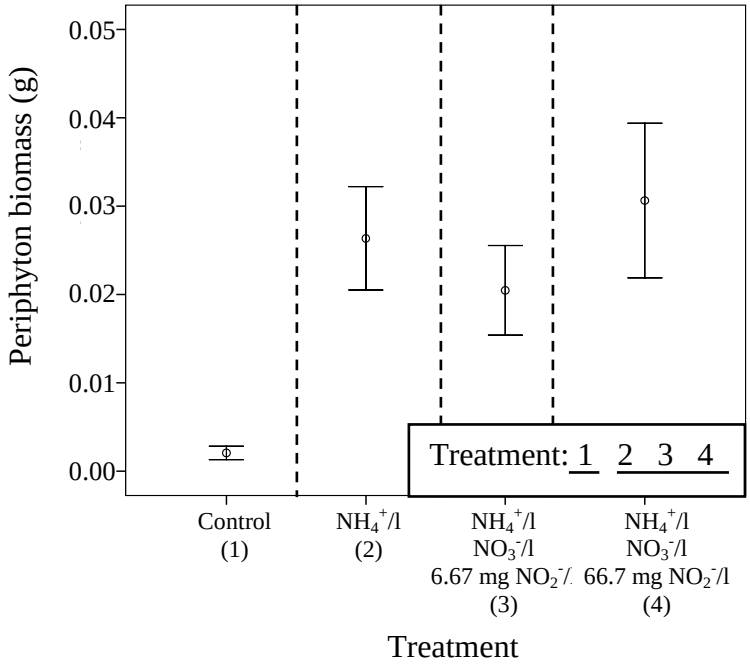


Fig. 4. Mass (mean ± 1 SE) of periphyton grown in the experimental tanks after 21 days of exposure to different levels of nitrogenous pollution. Treatment codes are shown in brackets. Results of HDS Tukey´s test (α= 5%) performed for the factor treatment are shown (common underbars notation).

Swimming performance

Neither swimming distance nor swimming speed were affected by treatments (Table 3). However, the exposure to the combination of high concentrations of nitrogenous compounds evidenced a reduction of mean swimming distance and treatments involving their mixture (treatments 3 and 4) showed a trend to reduce mean swimming speed (Fig.

5). Population of origin only affected distance swum when the effects of final mass were accounted for (Table 3). Larvae from C2 population swam lower mean distance than those from the rest of populations, although HDS Tukey´s test did not show significant differences among populations (Fig. 5). The interaction population x treatment was not significant in any case, revealing that the response of the larvae from the different populations to the treatments considered in the present study did not differ.

Table 3. Summary statistics for ANCOVAs on swimming variables for the common garden experiment. * P < 1%; ** P < 5%; *** P < 10%. NA: not applicable.

Variable	Source of variation	Not correcting for mass	Correcting for initial mass	Correcting for final mass
Distance	Mass	NA	$F_{1,195}$= 0.173	$F_{1,195}$= 6.128**
	Population of origin	$F_{2,196}$= 1.560	$F_{2,195}$= 1.621	$F_{2,195}$= 3.019***
	Treatment	$F_{3,12.031}$= 1.331	$F_{3,12.135}$= 1.370	$F_{3,13.845}$= 0.728
	Pool(Treatment)	$F_{12,196}$= 3.840*	$F_{12,195}$= 3.800*	$F_{12,195}$= 2.357*
	Population x Treatment	$F_{6,196}$= 1.069	$F_{6,195}$= 0.977	$F_{6,195}$= 1.028
Speed	Mass	NA	$F_{1,195}$= 1.720	$F_{1,195}$= 0.637
	Population of origin	$F_{2,196}$= 1.111	$F_{2,195}$= 1.133	$F_{2,195}$= 1.016
	Treatment	$F_{3,12.031}$= 2.201	$F_{3,12.131}$= 1.840	$F_{3,14.119}$= 1.414
	Pool(Treatment)	$F_{12,196}$= 3.827*	$F_{12,195}$= 3.916*	$F_{12,195}$= 2.062**
	Population x Treatment	$F_{6,196}$= 0.412	$F_{6,195}$= 0.537	$F_{6,195}$= 0.415

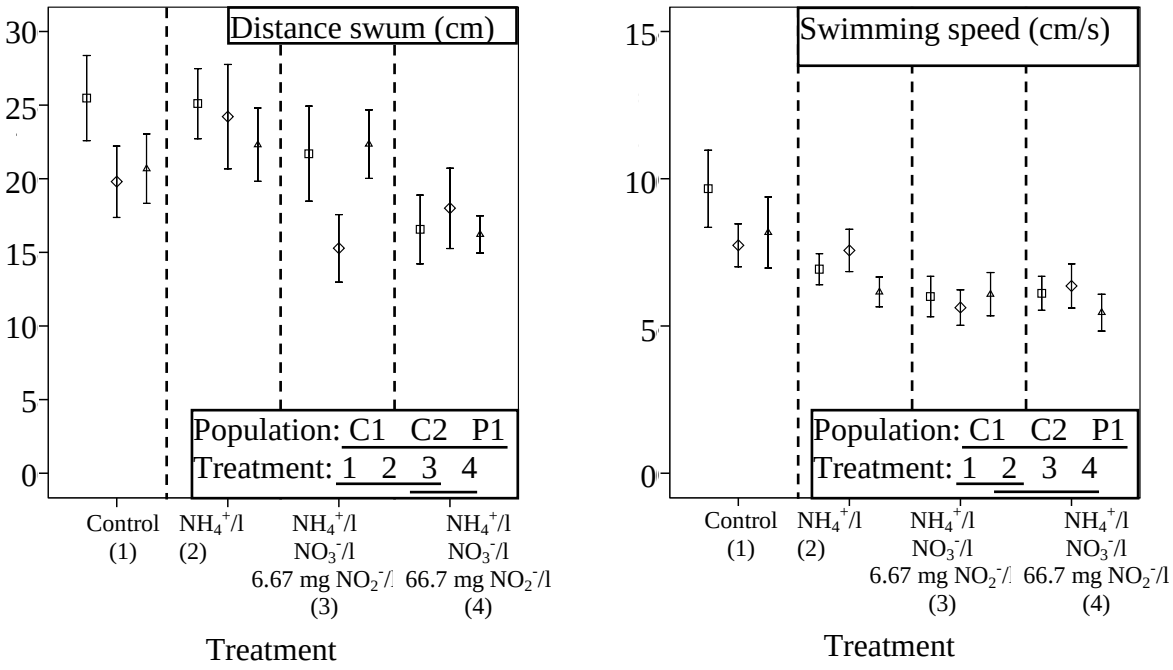


Fig. 5. Mean (± 1 SE) distance swum and larval speed after 21 days of exposure to different levels of nitrogenous pollution in the common garden experiment. Results of HDS Tukey´s test (α = 5%) for treatment are shown (common underbars notation). Treatment codes are shown in brackets. □ C1; ◇ C2; △ P1.

Trade-off between larval morphology and swimming performance

The analyses of homogeneity of slopes were only significant for the following relationships on total distance swum (Population x TL, $F_{2,\ 100} = 5.558$; P = 0.005, Treatment x BW, $F_{3,\ 100} = 4.016$; P = 0.01, and Treatment x BD, $F_{3,\ 100} = 9.346$; P= 0.0001) and swimming speed (Population x Treatment x TMW, $F_{6,\ 100} = 2.804$; P = 0.015). Separate stepwise multiple regression analyses performed for each source population and treatment revealed that the strength of the influence of morphological traits on swimming performance greatly varied across populations and treatments (Tables 4 and 5).

Table 4. Summary statistics for the regression analyses performed for distance swum and swimming speed (morphological traits: independent variables) for the common garden experiment. * P < 1%; ** P < 5%; *** P < 10%. See Material and Methods section for details on population and treatment codes. NM: not measurable.

Population	Treatment	Distance				Speed			
		Adjusted R^2	df (regression, residual)	F	P	Adjusted R^2	df (regression, residual)	F	P
C1	1	0.438	4, 14	4.510	0.015	0.000	NM, NM	NM	NM
	2	0.373	4, 14	3.673	0.030	0.445	1, 17	15.436	0.001
	3	0.929	7, 9	31.094	0.0001	0.742	4, 12	12.484	0.0001
	4	0.000	NM, NM	NM	NM	0.154	1, 14	3.740	0.074
C2	1	0.265	1, 18	7.857	0.012	0.564	3, 16	9.209	0.001
	2	0.388	3, 14	4.586	0.019	0.400	3, 14	4.780	0.017
	3	0.598	2, 15	13.631	0.0001	0.408	1, 16	12.716	0.003
	4	0.120	1, 15	3.174	0.095	0.204	2, 14	3.045	0.080
P1	1	0.286	5, 13	2.442	0.090	0.542	4, 14	6.326	0.004
	2	0.244	2, 16	3.898	0.042	0.438	3, 15	5.676	0.008
	3	0.207	1, 18	5.950	0.025	0.000	NM, NM	NM	NM
	4	0.139	2, 15	2.373	0.127	0.436	1, 16	14.141	0.002

Table 5. Coefficients (± 1 S.E.) for the morphological variables variables included in the regression analysis performed for distance swum and swimming speed for the common garden experiment. For clarity, only significant coefficients are shown. * P < 1%; ** P < 5%; *** P < 10%. See Material and Methods section for details on population and treatment codes. NA: not applicable.

Population	Treatment	BL	BW	TL	TD	TMW	Weight	BD	TMD
					Distance				
C1	1		-5.127 (1.249)*	3.531 (1.146)*			1.957 (0.731)**		-2.712 (1.168)**
	2	-2.820(1.003)**	-2.820(1.420)***	3.182(0.887)*				4.815(1.588)*	
	3	2.461(0.963)**	-3.021(0.643)*	2.493(0.781)**	-1.178(0.579)***	1.593(0.385)*	1.619(0.459)*	-5.159(1.088)*	
	4								
C2	1								2.615(0.933)**
	2					2.814(0.863)*	-1.386(0.537)**	4.309(1.427)*	
	3				-2.505(1.217)***		2.313(0.607)*		
	4	2.222(1.247)***							
P1	1	3.971(1.772)**		-1.538(0.850)***	4.806(1.820)**	-1.449(0.816)***		-5.675(2.518)**	
	2					-3.092(1.126)**	1.341(0.514)**		
	3						1.015(0.416)**		
	4						1.257(0.588)**	-2.858(1.474)***	
					Speed				
C1	1								
	2					1.274(0.324)*			
	3	-3.397(0.849)*	-2.938(0.714)*			1.533(0.389)*	2.132(0.480)*		
	4								1.044(0.540)***
C2	1	4.801(1.060)*				-2.408(0.563)*		-3.136(1.091)**	
	2	-3.496(1.647)***			2.931(1.243)**		0.730(0.338)**		
	3							2.687(0.753)*	
	4		-2.060(0.877)**				1.227(0.575)***		
P1	1		1.698(0.826)***	-1.855(0.635)**		1.543(0.536)**		-3.074(1.139)**	
	2					-2.046(0.637)*	0.763(0.308)**	1.750(0.849)***	
	3								
	4			1.921(0.511)*					

Trade-off between larval growth and swimming performance

Table 6 shows the results of the ANCOVAs performed to test the homogeneity of slopes for the growth-swimming analyses. The relationship between larval speed and growth differed across treatments. Growth positively influenced distance swum (Table 7; Fig. 6). As regards to speed, only control larvae showed a negative influence of growth (Table 7; Fig. 6).

Table 6. Summary statistics for the analyses of homogeinity of slopes (ANCOVAs) on swimming variables for the common garden experiment (covariables: larval growth) Absolute date are considered for both dependent variables and covariate.

Variable	Source of variation	d.f. numerator	d.f. denominator	F	P
Distance	Population of origin	2	184	0.141	0.869
	Treatment	3	187.585	0.626	0.599
	Pool (Treatment)	12	184	2.695	0.002
	Growth (mass)	1	184	1.738	0.189
	Population x Treatment	6	184	0.750	0.610
	Population x Growth	2	184	0.239	0.787
	Treatment x Growth	3	184	1.191	0.315
	Population x Growth x Treatment	6	184	0.789	0.580
Speed	Population of origin	2	184	0.328	0.721
	Treatment	3	183.683	2.535	0.058
	Pool (Treatment)	12	184	3.005	0.001
	Growth (mass)	1	184	1.769	0.185
	Population x Treatment	6	184	0.501	0.807
	Population x Growth	2	184	0.534	0.875
	Treatment x Growth	3	184	3.670	0.013
	Population x Growth x Treatment	6	184	0.577	0.748

Table 7. Summary statistics for the regression analyses performed on the swimming variables (dependent variables); larval growth (independent variable), for the common garden experiment. Since no growth x treatment or growth x population of origin interactions were detected for distance swum, data for this variable were pooled. Absolute date are considered for both dependent and independent variables. See Material and Methods section for details on treatment codes.

	Treatment	Regression analysis	B (S.E.)	P
Distance	—	Adjusted R^2= 0.063; $F_{1,56}$= 15.664; P= 0.0001	0.134 (0.034)	0.0001
Speed	1	Adjusted R^2= 0.076; $F_{1,56}$= 5.685; P= 0.021	-0.230 (0.097)	0.021
	2	Adjusted R^2= 0.166; $F_{1,54}$= 11.938; P=0.001	0.132 (0.038)	0.001
	3	Adjusted R^2= 0.037; $F_{1,53}$= 3.072; P= 0.085	0.088 (0.050)	0.085
	4	Adjusted R^2= 0.043; $F_{1,49}$= 3.259; P= 0.077	0.108 (0.060)	0.077

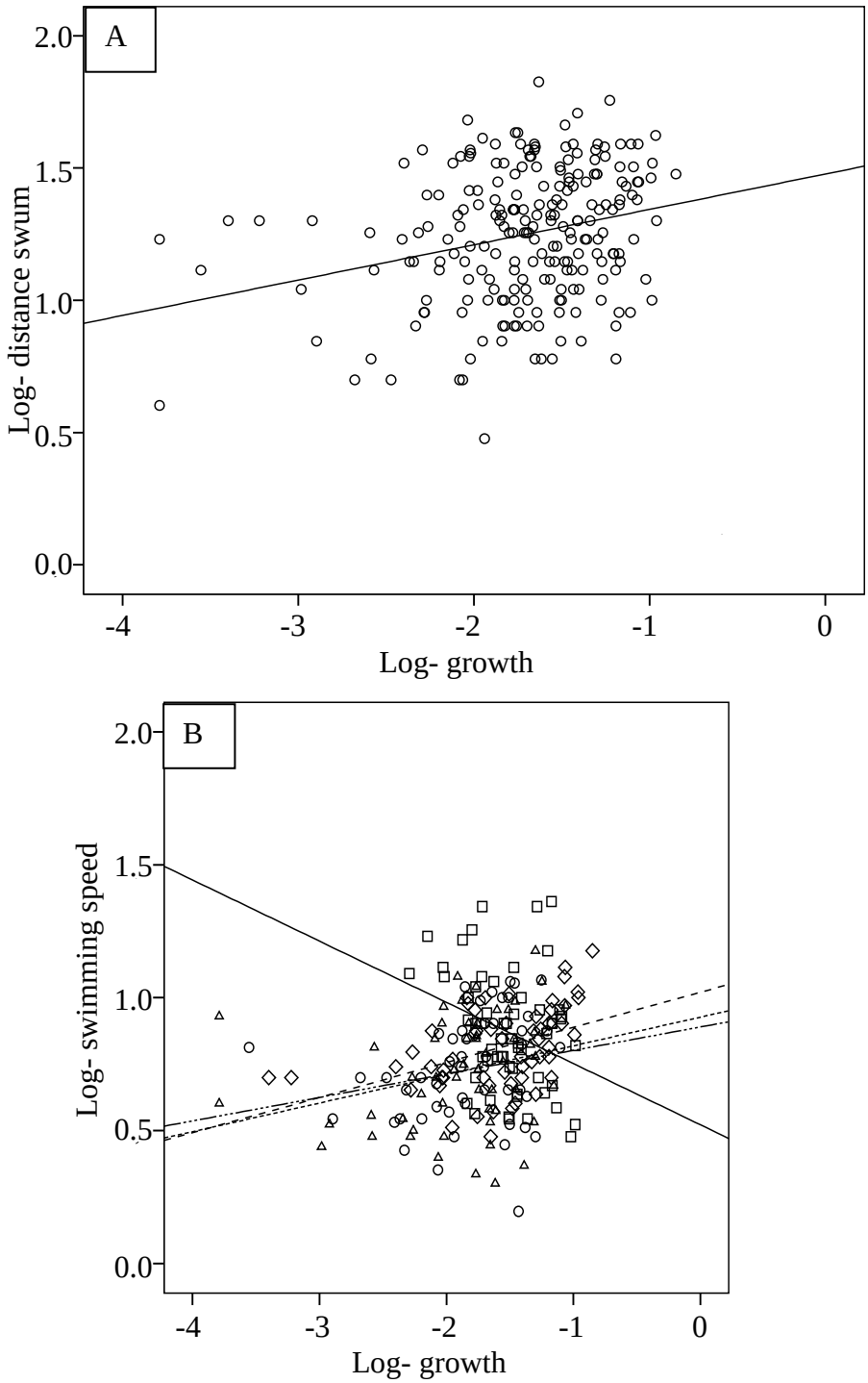


Fig. 6. Relationships between growth and distance swum (all treatments po oled) (A) and larval speed (B) for larvae exposed to different levels of nitrogenous pollution for 21 days in the common garden experiment. B)—□— Treatment 1 (control); - - ◇ - - - Treatment 2; — · · △ · · — Treatment 3; ····○···· Treatment 4 (see Material and Methods for details on treatment codes).

Field experiment

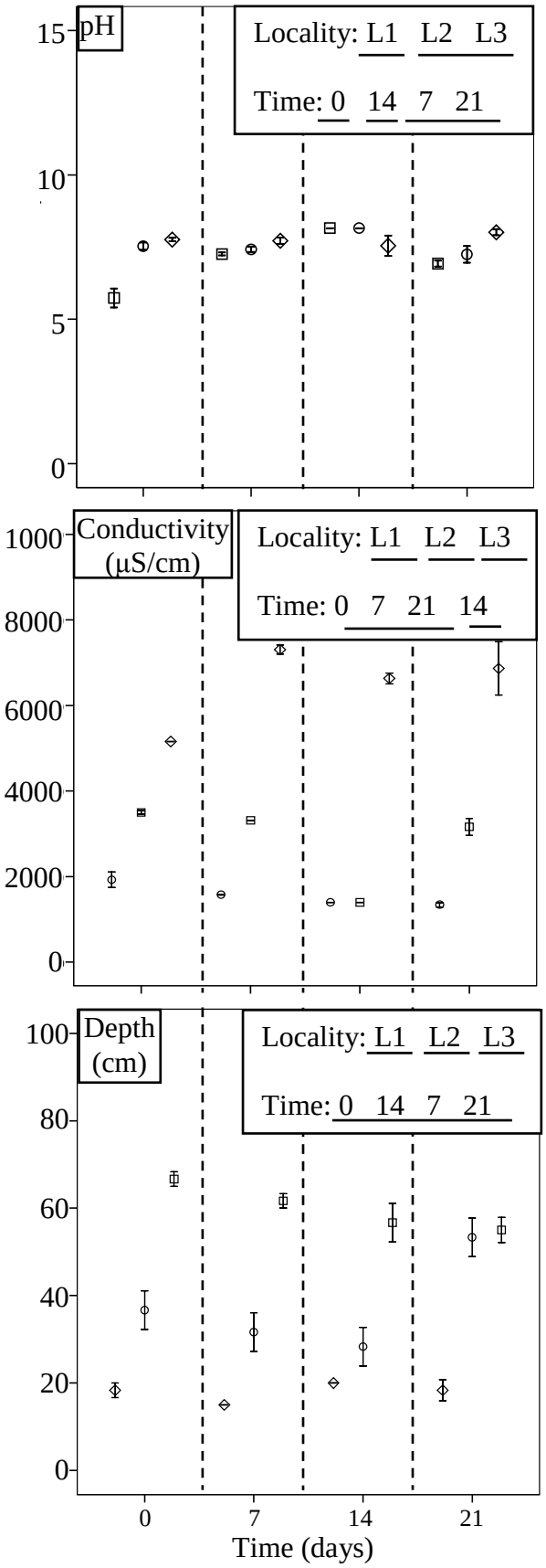
Water physicochemistry

Physicochemical characteristics of water were significantly affected by localities and time of measurement, which also affected the influence of the localities on water characteristics (Table 8). The lowest pH, conductivity and salinity levels were shown by L1 (pH) and L2 (conductivity and salinity), whereas L3 showed the highest values for all measured variables, with the exception of water depth (the lowest values for the studied localities) and pH (not differing with respect to L2) (Fig. 7). The significant time x experimental locality interaction recorded for all the physicochemical variables analysed (Table 8) indicates that differences among localities decreased during the experiment to increase again by its end, with the exception of water temperature and depth (Fig. 7).

As regards to nitrogenous ion concentration, the reduced number of cases for which detectable ammonium levels were recorded for any locality prevented its analysis. In relation to nitrite concentration, only L2 and L3 had reliable data and L3 showed higher nitrite concentration. Nitrate concentration was higher for L2 population. L1 showed the lowest value for nitrate concentration (Table 9; Fig. 8).

Table 8. Summary statistics of repeated measure ANOVA on physicochemical characteristics of water in the localities selected for the field experiment. df: degrees of freedom.

Variable	Source of variation	df	F	P
pH	Locality	2	18.691	0.0001
	Time	3	13.181	0.0001
	Time x Locality	6	11.536	0.0001
	Error	24		
Temperature	Locality	2	9.821	0.001
	Time	3	20.023	0.0001
	Time x Locality	6	7.351	0.0001
	Error	24		
Conductivity	Locality	2	987.642	0.0001
	Time	3	38.375	0.0001
	Time x Locality	6	37.351	0.0001
	Error	24		
Salinity	Locality	2	982.697	0.0001
	Time	3	43.605	0.0001
	Time x Locality	6	41.942	0.0001
	Error	24		
Depth	Locality	2	173.342	0.0001
	Time	3	3.126	0.045
	Time x Locality	6	4.420	0.004
	Error	24		



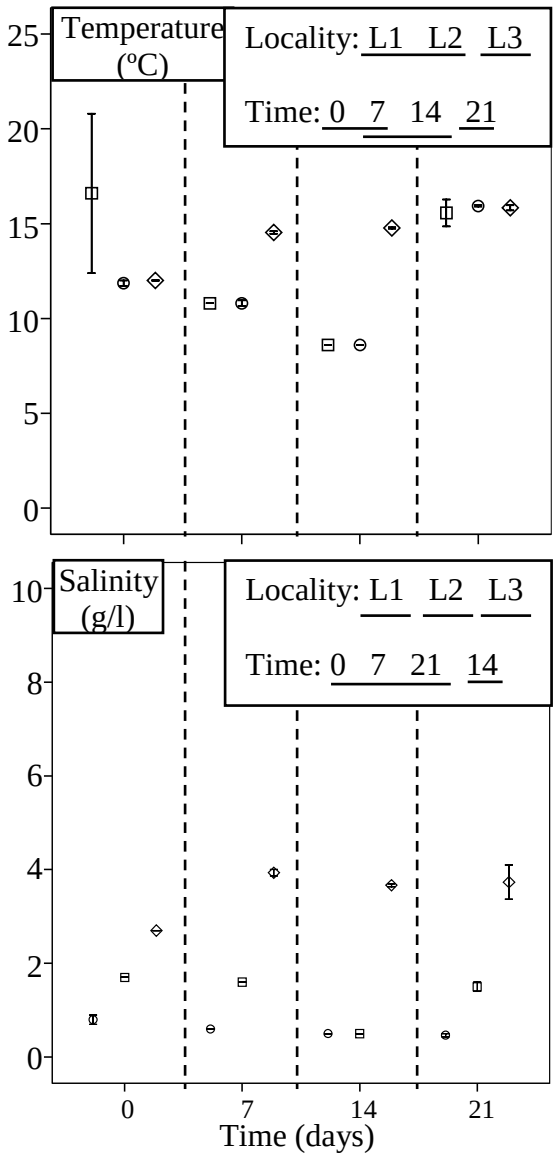


Fig. 7. Mean (±1 SE) for water physicochemical characteristics in the localities selected for the field experiment. Results of HDS Tukey´s test (α= 5%) performed for the factor treatment are shown (common underbars notation). Localities: □ L1;○ L2;◇ L3.

Table 9. Summary statistics of ANOVAs on nitrogenous ion concentration in the localities selected for the field experiment. NA: not detectable level of NH_4^+ in any location; *: not detectable NO_2^- in L1 locality only L2 and L3 localities could be compared. ndf: numerator degrees of freedom; ddf: denominator degrees of freedom.

Variable	Source of variation	nddf	ddf	F	P
Ammonium	Locality	NA	NA	NA	NA
Nitrite	Locality	1*	4	9.009	0.040
Nitrate	Locality	2	8	254.824	0.0001

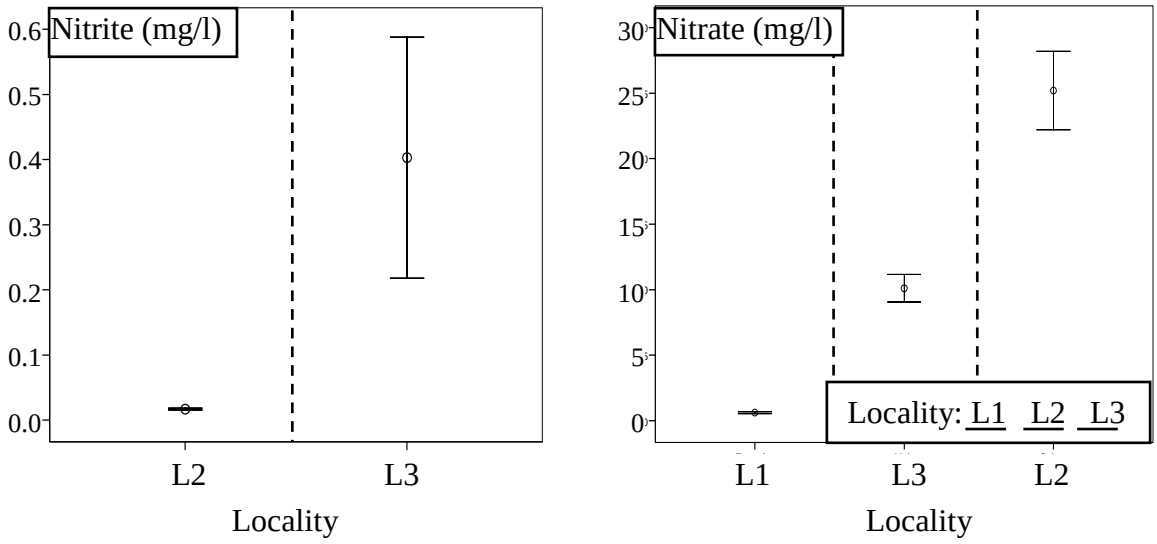


Fig. 8. Mean (±1 SE) for nitrite and nitrate concentrations (mg/l) in the localities selected for the field experiment. Results of HDS Tukey´s test (α = 5%) for treatment are shown (common underbars notation).

Swimming performance

Neither swimming distance nor swimming speed were affected by population of origin or locality. Additionally, the swimming performance of the larvae from the different populations did not differ at different experimental localities (Table 10; Fig. 9).

Table 10. Summary statistics for ANCOVAs on swimming variables for the field experiment. * P<1%; ** P<5%; ***P<10%. NA: not applicable.

Variable	Source of variation	Not correcting for mass	Correctig for initial mass	Correcting for final mass
Distance	Mass	NA	$F_{1,43}$= 0.453	$F_{1,43}$=0.170
	Population of origin	$F_{2,44}$= 2.206	$F_{2,43}$= 1.968	$F_{2,43}$=2.022
	Locality	$F_{2,5.850}$= 0.328	$F_{2,7..551}$= 0.100	$F_{2,9.202}$=0.445
	Enclosure(Locality)	$F_{6,44}$= 0.896	$F_{6,43}$= 0.948	$F_{6,43}$=0.796
	Population x Locality	$F_{4,44}$= 0.406	$F_{4,43}$= 0.497	$F_{4,43}$=0.349
Speed	Mass	NA	$F_{1,43}$= 0.798	$F_{1,43}$= 0.005
	Population of origin	$F_{2,44}$= 0.441	$F_{2,43}$= 0.056	$F_{2,43}$= 0.411
	Locality	$F_{2,5.848}$= 2.329	$F_{2,8.557}$= 1.263	$F_{2,9.161}$= 1.408
	Enclosure(Locality)	$F_{6,44}$= 0.886	$F_{6,43}$= 0.591	$F_{6,43}$= 0.806
	Population x Locality	$F_{4,44}$= 0.501	$F_{4,43}$= 0.311	$F_{4,43}$= 0.447

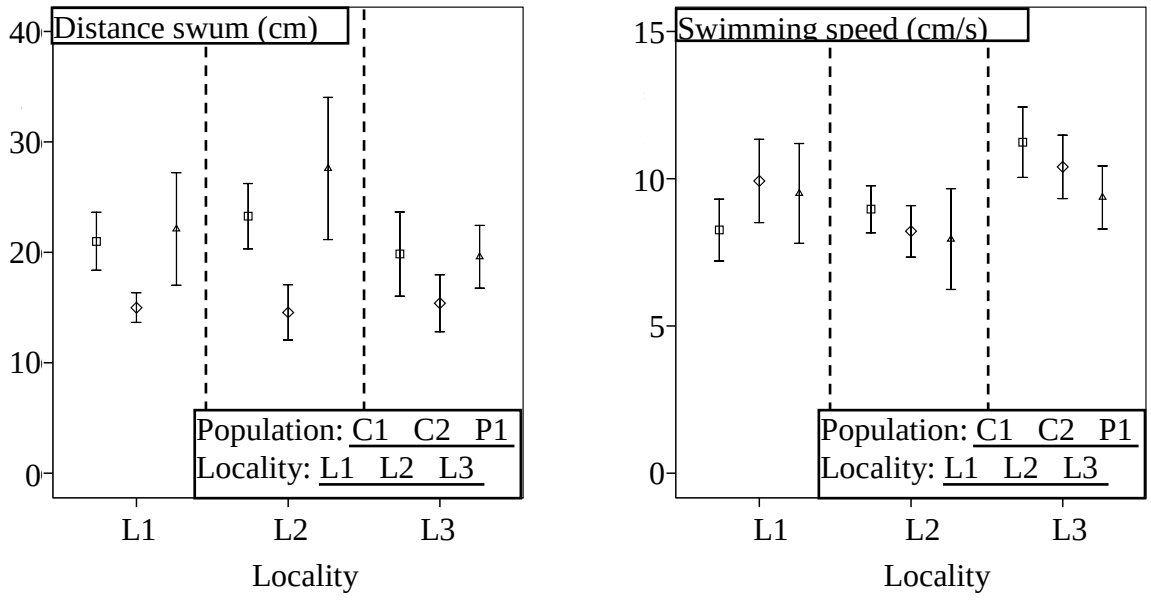


Fig. 9. Mean (± 1 SE) distance swum and larval speed after 21 days of exposure to field localities in the field experiment. Results of HDS Tukey´s test (α = 5%) for treatment are shown (common underbars notation). Source populations: □ C1; ◇ C2; △ P1.

Trade-off between larval growth and swimming performance

The influence of growth on swimming only differed across population and locality for distance and speed, respectively (Table 11). As regards distance, separate analysis performed for each population revealed that only existed a significant influence of growth on this variable for larvae from C2 population (Table 12). Higher growth supposed higher distances (Fig. 10). In relation to speed, a significant influence of growth was detected only for L2, for which the relationship had a positive sign (Table 12; Fig. 10).

Table 11. Summary statistics for the analyses of homogeinity of slopes (ANCOVAs) on swimming variables for the field experiment (covariables: larval growth). Absolute date are considered for both dependent variables and covariate.

Variable	Source of variation	d.f. numerator	d.f. denominator	F	P
Distance	Population of origin	2	35	2.926	0.067
	Locality	2	36.544	0.036	0.965
	Enclosure (Locality)	6	35	1.548	0.192
	Growth (mass)	1	35	0.399	0.532
	Population x Locality	4	35	1.282	0.296
	Population x Growth	2	35	3.542	0.040
	Locality x Growth	2	35	0.099	0.906
	Population x Growth x Locality	4	35	1.235	0.314
Speed	Population of origin	2	35	0.572	0.569
	Locality	2	35.805	4.402	0.020
	Enclosure (Locality)	6	35	0.782	0.590
	Growth (mass)	1	35	1.068	0.308
	Population x Locality	4	35	1.504	0.222
	Population x Growth	2	35	0.529	0.594
	Locality x Growth	2	35	3.945	0.029
	Population x Growth x Locality	4	35	1.414	0.250

Table 12. Summary statistics for the regression analyses performed on the swimming variables (dependent variables); larval growth (independent variable), for the field experiment. Absolute date are considered for both dependent and independent variables. * Regression analysis excluded independent variable from the regression model, which consisted only in the constant.

	Population	Regression analysis	B (S.E.)	P
Distance	C1*	Adjusted R^2= 0.000; $F_{0,21}$= -; P= -	-	-
	C2	Adjusted R^2= 0.190; $F_{1,17}$= 5.214; P= 0.036	0.349 (0.153)	0.036
	P1*	Adjusted R^2= 0.000; $F_{0,17}$= -; P= -	-	-
	Locality			
Speed	L1	Adjusted R^2= 0.098; $F_{1,21}$= 3.397; P= 0.079	-0.270 (0.146)	0.079
	L2	Adjusted R^2= 0.203; $F_{1,17}$= 5.597; P= 0.030	0.313 (0.132)	0.030
	L3*	Adjusted R^2= 0.000; $F_{0,16}$= -; P= -	-	-

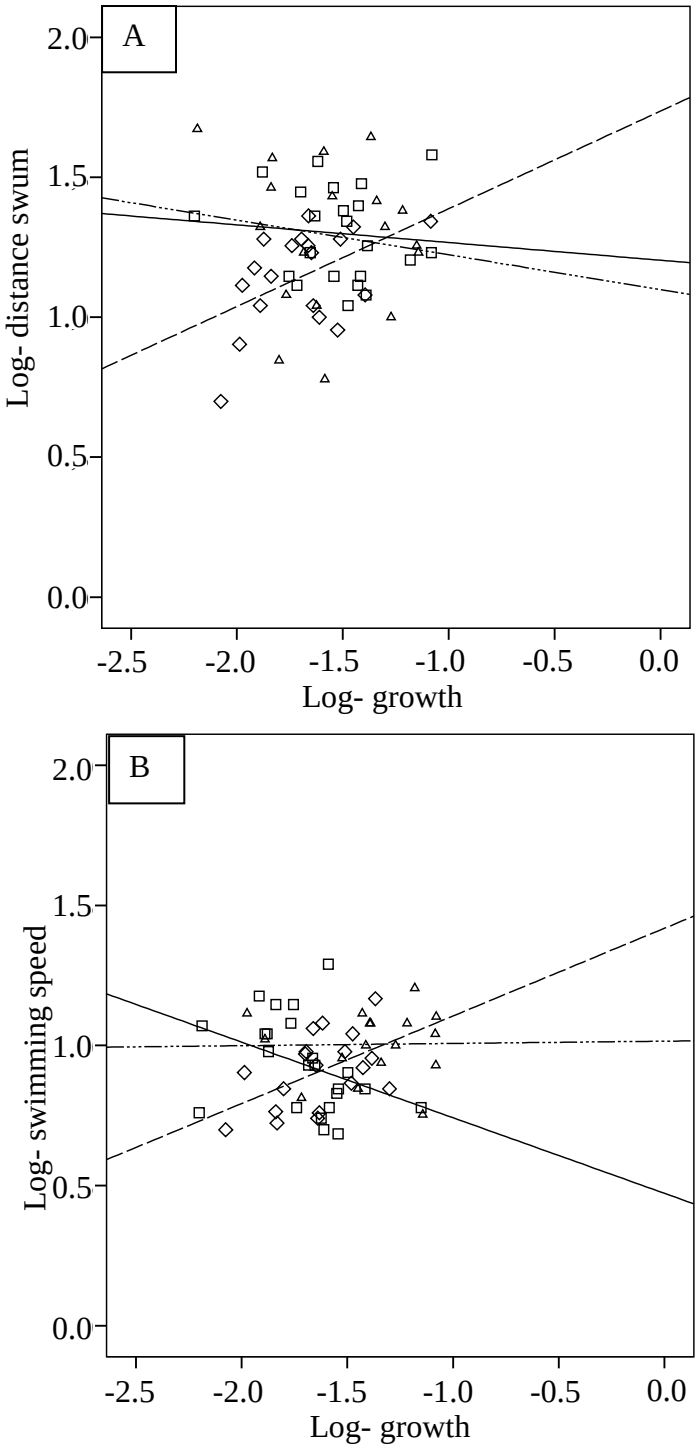


Fig. 10. Relationships between growth and distance swum (A) and larval speed (B) for larvae exposed to different field localitites for 21 days in the field experiment. A) Source population: —□— C1; - - ◇ - - C2; — ··△·· — P1; B) Experimental locality: —□— L1;- - ◇ - - L2; — ··△·· — L3 (see Material and Methods for details on source population and experimental locality codes).

DISCUSSION

The results obtained evidenced that the exposure to the combination of nitrogenous compounds negatively affected larval performance. However, neither swimming distance nor swimming speed were affected by nitrogenous pollution at both venues (mesocosm and field experiment) when larval mass was taken into account. This result disagrees with previous analyses with other pollutants (Jung & Jagoe, 1995; Bridges, 1997; but see Widder & Bidwell, 2006, 2008). Our previous laboratory experiments showed that the exposure to nitrogenous compounds affected spontaneous activity and morphology (including tail traits) of *P. perezi* larvae (Egea-Serrano *et al.*, chapters 5 and 7 in the present thesis). Therefore, we would expect a significant effect of both treatments and differentially polluted field localities.

Burst speed is assumed to be correlated with greater survival in larval anurans (Jung & Jagoe, 1995) and some direct evidences corroborate this prediction (Watkins, 1996; Kaplan & Phillips, 2006). Therefore if nitrogenous pollution can reduce locomotor performance, we would expect a reduction in fitness for those populations exposed to contaminants. Nitrite can affect the enzyme activities responsible for the transmission of nerve impulses in fishes (Das *et al.*, 2004) that indirectly may limit locomotor performance. Therefore we could expect a decline in tadpole locomotor performance in polluted areas. However, our results (when correcting for larval mass) does not support this hypothesis due likely to the effect of nitrogenous pollutants on enzyme activity was not intense enough to affect larval motor skills, as Widder and Bidwell (2006, 2008) suggested to explain a lack of effect of chlorpyrifos on swimmig speed. Further physiological research is needed to understand the pathways underlying

the lack of effect of nitrogenous pollution on swimming performance of larvae of *P. perezi*.

The studied populations may have been exposed to different nutrient concentration (Vidal-Abarca *et al.*, 2000; Ballester, 2003) due to they are located in contrasting environments as regards their pollution level. Hence, an adaptive process would be expected, as it has been previously mentioned for *Rana temporaria* and *P. perezi* (Johansson *et al.*, 2001; Egea-Serrano *et al.*, 2009). However, the lack of significant population of origin x treatment (or locality) interactions suggests that the response detected is consistent across populations. The results obtained evidence that individual susceptibility to predators may not be affected by the effects of nitrogenous pollution on swimming performance, since larval escape ability is not modified neither by treatments nor by environment. Nevertheless, previous assays (Egea-Serrano *et al.*, chapter 5 in the present thesis) showed that both activity level and habitat use by larval *P. perezi* were modified by the exposure to nitrogenous treatments in the laboratory. The increased activity level and number of movements through the water column this study reported suggest that predator encounter rates would increase as a consequence of the exposure to pollution, which may represent an increased risk of predation, although larvae escape abilities may not differ as regards to not polluted environments.

Morphological variables affected swimming capabilities and the sign of their influence is greatly specific for each population of origin and treatment. Dayton *et al.* (2005) stated that tadpole swimming abilities are affected by thrust-propelling regions. Moreover, the influence of body and, more specifically tail morphology, on swimming speed has been described for the study species in previous settings (Tejedo *et al.*, unpublished data). So, a positive influence of tail length or tail depth on swimming speed would have been expected. Nevertheless, the great variation obtained disagrees

with this hypothesis. This scenario points out the relevance of taking into consideration the environment, as well as the particular characteristics of a studied population when analysing locomotor capabilities.

A significant trade-off between growth and swimming performance has been detected for both the common garden and the field experiments. Regarding swimming speed, Arendt (2003) stated that a negative influence of growth on swimming speed would be a general situation for different organisms, such as fishes and amphibians. Billerbeck *et al.* (2001) showed that higher growth rates represent lower available energy to locomotion. This fact, joined to the higher energetic demand that detoxification pathways may represent (Wright & Wright, 1996), would make us to expect a more marked negative relatioship between growth and speed when larvae were exposed to pollution both in mesocoms and in the field. However, the expression of the negative trade-off between growth and swimming performance was only confirmed in the control treatment. The exposure either to high concentration of ammonium acting isolated (treatment 2, 13.5 mg NH_4^+/l) or to polluted permanent rivers (i.e. L2) produced a significant positive trade-off between swimming speed and growth. Egea-Serrano *et al.* (chapter 7 in the present thesis) described that this treatment and experimental locality did not affect larval growth, whereas the combination of nitrogenous compounds (i.e. treatments 3 and 4) and other polluted permanent rivers (L3) did. This fact would suggest that the exposure to pollutants not affecting larval growth may positively affect the relationship between swimming speed and growth, whereas such trade-off would disappear when growth is affected by pollution, both positively and negatively. Arendt (2000) found out that growth rate may affect muscle development. The results obtained suggest that the capability to escape from predators depends on how to get larval size, and that the costs of rapid growth are environmentally dependent.

Although the causes modifying the influence of growth on distance swum has not been described, it makes sense to consider that factors influencing such relationship would be the same that those conditioning the influence of growth on larval speed. Nevertheless, the influence of growth on such parameter did not show the same trend that swimming speed. Bearing in mind that both growth and speed are composite variables, is likely that several underlying factors may be affecting the trade-off (Arendt, 2003).

To summarize, the results obtained suggest the lack of direct effect of nitrogen pollution on swimming performance of larval *P. perezi*. Nevertheless, the exposure to nitrogenous pollutants may produce indirect deleterous effects on larvae through the influence of morphology on swimming parameters, as well as the trade-offs between growth and swimming speed. These considerations points out the relevance of considering trade-offs with morphology and growth when studying swimming performance and of performing detailed physiological studies which would lead to a more complete understanding of the mechanisms underlying the impact of nitrogenous pollution on amphibian larvae swimming abilities.

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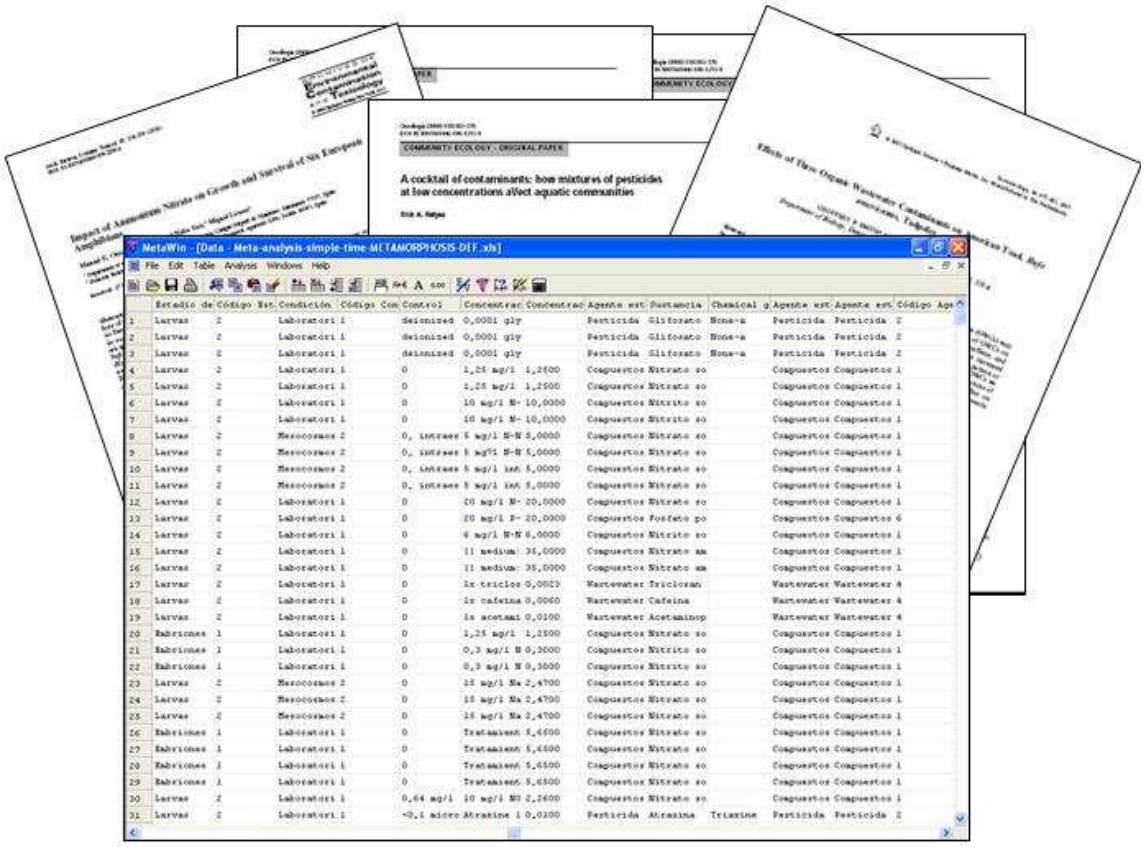
BLOQUE IV

ANÁLISIS DE LOS EFECTOS DE LA CONTAMINACIÓN SOBRE LOS ANFIBIOS



CAPÍTULO 9

ARE AMPHIBIANS ACTUALLY THREATENED BY CHEMICALS? A META-ANALYTIC REVIEW

Abstract. Many studies have assessed the impact of a great variety of pollutants on many amphibian populations in different experimental venues. The analysis of bibliographic reviews by vote counting methods has described pollution as one of the major threats amphibians are facing nowadays. However, because the poor statistical power of these methods, as well as the impossibility of determining the magnitude of the effect and of comparing the responses among previously defined groups, further research is needed to get a global perspective of the actual impact of pollution on amphibians. We conducted a meta-analysis of experimental studies that measured the effects of different kinds of chemical pollutants on amphibian survival, length, weight, time to hatching, time to metamorphosis and rate of abnormalities. With the exception of time to hatching and time to metamorphosis (for which no significant effect was found), the exposure to pollutants had a significant negative impact on survival, size and abnormality rates. Despite these overall effects, effect sizes varied greatly among the categories for the groups defined *a priori*. No phylogenetic signal was detected for the studied variables, with the exception of time to hatching. This result would hint that related species are not more sensitive to pollutants than unlike taxa, and sensitivity variation to contaminants is independent of the phylogeny. Some level of publication bias was recorded for those variables for which no significant effect size was detected (time to hatching and to metamorphosis). We conclude that the impact of pollution on amphibians is moderate to largely negative, which implies that pollution is an important threat and may be a cause of the present amphibian biodiversity crisis.

Key words: Amphibians, chemical pollutants, meta-analysis, fitness-related traits, phylogenetic autocorrelation, synergy

INTRODUCTION

Significant demographic fluctuations within amphibian populations have been reported (Pechmann *et al.*, 1991; Tejedo, 2003). Although such oscillations may be attributed to natural trends, the negative impact of anthropogenic activities on amphibians is becoming increasingly conspicuous. The dispersion of emergent diseases, habitat destruction, introduction of exotic species and the pollution of both terrestrial and aquatic habitats have been described as important threatening factors (Stuart *et al.*, 2004). Thus, the determination of the influence and magnitude of their effects on amphibian populations is of great relevance to develop proper management strategies.

Habitat pollution may be due to a great variety of chemical compounds, such as fertilizers, pesticides, heavy metals or even road de-icers. This broad array of pollutants is increasingly present in the environment by direct application, run off from crop fields or mining, urban and industrial sewage or by atmospheric deposition (e.g. Vitousek *et al.*, 1997; Linder & Grillitsch, 2000; Sparling, 2000; Ritter & Bergstrom, 2001). Therefore, their presence is widespread (Carpenter *et al.*, 1998) and is expected to increase in the near future (Tilman *et al.*, 2001; Galloway *et al.*, 2003).

As regards amphibians, chemical pollutants have been reported to affect biotic properties of individuals as relevant for fitness such as survival, size or development (e.g. Ortiz *et al.*, 2004; Griffis-Kyle, 2007). Moreover, different degree of toxicity would be expected among pollutants due to the existing great diversity of chemical compounds. Additionally, because of human activities, factors such us pathogenic organism or ultraviolet-B radiation are increasingly common in natural environments (Daszak *et al.*, 2001; McKenzie *et al.*, 2003). Since these stressing factors and chemical

pollutants may be found in the same habitat, analysing the interaction among these stressors is of great relevance when considering the effects of pollution on amphibians.

To date, most studies dealing with the impact of pollutants on amphibians have been performed in laboratory conditions (Boone & James, 2005). Although these studies may have used ecologically relevant concentrations, the results obtained in such conditions may not be applicable to more natural conditions (Boone & Bridges, 2003). Since actual concentrations in the environment may be affected by several factors such as plant uptake, denitrification or sediment trapping (e.g. Ritter & Bergstrom, 2001), it is possible that those studies performed in the laboratory overestimated the impact of chemical pollutants on amphibians (Boone & Bridges, 2003). This consideration emphasizes the need of further studies that contrast whether pollution harmful effect differs between experimental venues (Skelly, 2002).

Furthermore, the impact of pollutants such as fertilizers or pesticides at the organismal level may vary with the developmental stage at which individuals are initially exposed (Bridges, 2000, Greulich & Pflugmacher, 2003; Griffis-Kyle, 2005; Ortiz-Santaliestra *et al.*, 2006). Moreover, inter- and intra-specific variations in the tolerance to pollutants have been described (Marco *et al.*, 1999; Shinn *et al.*, 2008; Snodgrass *et al.*, 2008). All these aspects point to the relevance of taking into consideration a great number of moderating variables to analyse the impact of chemical pollutants on amphibians.

To get a global perspective of the actual impact of chemical pollution on amphibians, a number of bibliographic reviews have been published (Cowman & Mazanti, 2000; Linder & Grillitsch, 2000; Sparling, 2000; Camargo *et al.*, 2005; Relyea & Hoverman, 2006; Marco & Ortiz-Santaliestra, in press). These reviews present a list of effects based on the statistical significance of each study. However, the conclusions

obtained using vote counting methods may be not highly accurate or their estimates may be highly biased, since they have poor statistical power (Rosenberg *et al.*, 2000) and do not provide any reliable way of both determining the magnitude of the effect and of comparing the responses among groups previously defined (Gurevitch *et al.*, 2000). An alternative methodology to vote counting methods is provided by meta-analysis techniques. These analyses have been successfully used to identify the overall effects of stressing factors on amphibians (e.g. Bancroft *et al.*, 2007) and to synthesize factorial data (Gurevitch *et al.*, 2000), avoiding the limitations and subjectivity of traditional reviews.

The main objectives of the present study were: 1) to determine the overall effect of chemical pollutants on amphibians through meta-analytic techniques; 2) to assess the effects of the interaction of pollutants and other stressors on amphibians; 3) to analyse the existence of significant differences among the categories for groups defined *a priori* (i.e. whether pollutants effects differ across amphibian lineages, experimental venues, developmental stages and type of pollutant).

MATERIAL AND METHODS

Data colection

To analyse patterns of effects of pollutants on amphibians, several methods were used to identify the studies to include in the present analyses. First, we searched four electronic databases (ISI Web of Science, BIOSIS Previews, ScienceDirect, Scirus) for the words: nitrate, nitrite, ammonium, pesticides, heavy metals, for dates earlier than 2008. Second, we examined the citations from two recent reviews (Camargo *et al.*,

2005; Marco & Ortiz-Santaliestra, in press), as well as from the rest of the studied bibliographic references on this topic. Third, we included several unpublished datasets.

The studies obtained were analysed and included in the meta-analyses whether they met the following criteria. First, the articles had to report data on amphibian survival, time to hatching, time to metamorphosis, total length, weight or abnormality rate. Second, the studies had to state that concentrations used in the experiments were ecologically relevant for the location. Third, the studies had to clearly give mean values, sample size and a measure of error for the response variables mentioned above for both a control group (i.e. not exposed to pollution) and an experimental group (i.e. exposed to pollution). Fourth, studies that combined pollution effects with other factors (e.g. pollution with resource competition, pollution with predators, etc) without presenting separate results were excluded. In the case that the selected publications reported data for more than one species, population, pollutant or pollutant concentration, all of them were considered to be independent and included in the meta-analyses.

To conduct meta-analyses, mean, standard deviation (SD) and sample size (n) were obtained for both the control and the experimental group. When means and measures of error were presented graphically, the plot was digitized and ImageProPlus software was used to estimate values. If standard errors (SE) were reported, these were transformed according to the equation: $SD = SE \cdot \sqrt{n}$. For those studies which did not clearly show or include the required data, we attempted to contact authors to obtain the missing data.

In addition we also compiled information regarding family, developmental stage (embryos, larvae or metamorphs), experimental venue (laboratory, mesocosm or enclosure experiments, as well whether the animals were collected in the field) and kind of pollutant (nitrogenous compounds, phosphorous compounds, pesticides, road de-

icers, heavy metals and other watewater contaminants [i.e. perchlorate, boron, acetaminophen, caffeine and triclosan]).

To assess the effect of the combination of different types of stressing factors (both biotic and abiotic), a factorial meta-analysis was conducted (Gurevitch *et al.*, 2000). The original objective was to examine the effect of the interaction between pollutants and other stressors. However, since several original studies reported the impact of the combination of different types of pollutants, the effect of this interaction could not be discarded. Therefore, because the group dealing with other stressors included also pollutants, it did not make sense to differentiate the effect of these compounds from that of other stressors. Consequently, the denomination *first group of stressing factors* (FGSF) (nitrogenous compounds, pesticides and wastewater pollutants) and *second group of stressing factors* (SGSF) (competitors, pH, predators, UV radiation, other wastewater pollutants and mold) was used. The factorial meta-analysis examines the magnitude of the main effects of FGSF, SGSF and their interaction. Data obtained from the publications meeting the above criteria and showing a 2 x 2 factorial structure were organized into four treatments combinations (Gurevitch *et al.*, 2000): 1) absence of both FGSF and SGSF; 2) presence of FGSF in the absence of SGSF; 3) presence of SGSF in the absence of FGSF; 4) presence of both FGSF and SGSF (Fig. 1). Due to the scarcity of experiments for the rest of parameters, factorial meta-analysis could only be performed for survival. For each experiment, mean, standard deviation and sample size were obtained for both the control and the experimental group.

Data analysis

Meta-analysis.- For all studies with two treatments (pollutant absence versus pollutant presence), Hedge´s d^+ was used as the metric of standardized effect size for the studied

variables. Hedge´s d^+ provides a measure of the overall magnitude of the treatment effect, adjusting for small sample sizes. Since the absence of pollutant was considered as control, negative effect sizes would indicate pollutant induction of reduced survival, length, weight, time to hatching, time to metamorphosis and abnormality rate.

For each trait studied, data were analysed using random effects models to calculate the grand mean effect size. Additionally, any difference among *a priori* defined groups was analysed using mixed-effects models. Such groups included family, developmental stage (embryonic, larval o metamorphic individuals), experimental venue (laboratory, mesocosm or field experiments, as well whether the animals were collected in the field) and kind of pollutant (nitrogenous compounds, phosphorous compounds, pesticides, road de-icers, heavy metals and other watewater contaminants [i.e. perchlorate, boron, acetaminophen, caffeine and triclosan]). When mixed-effects models were ran, mean effect sizes and 95% confidence limits for each class were calculated. Additionally, heterogeinity statistics were calculated to quatinfy both between-group (Q_B) and within-group (Q_W) variation. The magnitude of the overall effect size is generally interpreted as "small" if $d^+ = 0.2$, "medium" if $d^+ = 0.5$, "large" if $d^+ = 0.8$, and "very large" for values of $d^+ > 1.0$ (Cohen, 1969). Effect sizes were considered significant if 95% confidence intervals did not cross zero. Effect sizes within analyses (e.g., nitrogenous compounds effect vs. pesticides effect) were considered different from one another if their 95% confidence intervals did not overlap. All statistical analyses were performed using MetaWin 2.1 statistical program.

Factorial meta-analysis.- Since many studies had a two stressful factors acting in combination and framed in a factorial experimental design we examine whether the effect of a first stressors behave additively or not when a second stressor was added. As

in the previous analyses, Hedge´s d^+ standardized effect size and its corresponding sampling variance were calculated with modifications due to the factorial design of the experiments, following the calculations developed by Gurevitch *et al.* (2000). For each study, the following statistics were calculated: 1) the mean effect size for the exposure to FGSF, when SGSF was present (dp, s) and when it was absent (dp, ns); 2) the mean effect size for the exposure to SGSF, when FGSF was present (ds, p) and absent (dnp, s); 3) the average effect of the exposure to FGSF (dp) or to SGSF (ds); 4) their interaction (di) (see Fig. 1 for a representation of the experimental treatments and procedures used to calculate the statistics described). Positive effects of the exposure to FGSF across SGSF are revealed when dp>0. Analogously, positive value for ds indicates a positive effect of the exposure to SGSF across FGSF. A negative value of di indicates that the presence of FGSF has a greater effect on amphibian survival when it is combined with SGSF.

		SECOND GROUP OF STRESSING FACTORS (SGSF) (competitors, pH, predators, UV radiation, other wastewater pollutants and mold)	
		PRESENCE (s)	ABSENCE (ns)
FIRST GROUP OF STRESSING FACTORS (FGSF) (nitrogenous, pesticides, wastewater pollutants)	PRESENCE (p)	1 p, s	2 p, ns
	ABSENCE (np)	3 np, s	4 np, ns

Individual effects

FGSF presence with presence of SGSF (dp, s): 1 – 3

FGSF presence with absence of SGSF (dp, ns): 2 – 4

SGSF presence with FGSF presence (dp, s): 1 – 2

SGSF presence with No FGSF presence (dnp, s): 3 – 4

Main effects

FGSF (dp): (1 + 2) - (3 + 4);

SGSF (ds): (1 + 3) – (2 + 4);

Interaction FGSF-SGSF (di- contrast FGSF presence in the presence of SGSF minus FGSF presence in the absence of SGSF): (1 - 3) – (2 - 4).

Fig. 1. Design of the 2 x 2 factorial meta-analysis with all orthogonal manipulations of a first group of stressing factors (p) (nitrogenous, pesticides, wastewater pollutants) and a second group (s) (competitors, pH, predators, UV radiation, other wastewater pollutants and mold). Individual effects refer to the effects of pollutant presence with and without other stressors presence, as well as to the effects of these stressors under absence and presence of pollutants. Main effects refer to the average effect of pollutants, other stressors presence and their interaction across environments. Effect size estimations follow the calculations expressed with the number labels of each treatment. These formulae represent the numerator in the effect size calculation equations given by Gurevitch *et al.* (2000).

Phylogenetic comparative analysis.- To determine whether phenotypic plasticity induced by pollutants (i.e. effect size) is significantly associated with the phylogenetic history, we conducted tests for serial independence (TFSI) on continuous characters (Abouheif, 1999) for each response variable studied. The diagnosis is based on a measurement of the autocorrelation of a trait across phylogeny, in the form of a *C*-statistic, resulting from similarity between adjacent phylogenetic observations. The topology and associated numerator distribution was randomized 2,000 times and the *C*-statistic was calculated for each randomized topology to build the null hypothesis. The observed *C*-statistic was compared to the randomized distribution to calculate its level of significance. Significant phylogenetic autocorrelation was defined when the observed *C*-statistics falls to the right of the distribution of the randomized *C*-statistics and $P \leq 5\%$. Such a result would imply that related species show similar response regardless the effect of pollutants.

The analyses were conducted after calculating a single effect size for each species. To conduct the phylogenetic analyses, a topology was constructed following Frost *et al.* (2006). To establish the phylogenetic relationships among species within families, Frost *et al*.'s (2006) phylogenetic hypothesis was combined with additional detailed phylogenetic assessments: Salamandridae (Zajc & Arntzen, 2000; Weisrock *et al.*, 2006; Zhang *et al.*, 2008), Ambystomatidae (Shaffer *et al.*, 1991; Jones *et al.*, 1993), Pelobatidae and Pelodytidae (García-París *et al*., 2003), Myobatrachidae (Schäuble *et al*., 2000; Read *et al*., 2001), Hylidae (Faivovich *et al*., 2005), Bufonidae (Pauly *et al*., 2004), and Ranidae (Veith *et al*., 2003; Hillis & Wilcox 2005; Scott 2005) (Appendix 1). Branch length information was not available for our composite phylogenies, but all analyses performed can be run without knowledge of branch lengths, using only

topology. Before conducting the phylogenetic analyses, the same value to all branch lengths was assigned.

Publication bias.- For each studied variable, for both simple and factorial meta-analyses, a weighted fail-safe number was calculated using a fail-safe number calculator that is applicable to random-effect models (Rosenberg, 2005; http://www.public.asu.edu/~mrosenb/software.html#failsafe). The results obtained are considered robust when the fail-safe number is larger than 5n + 10 (n= the number of studies) (Rosenberg, 2005). Additionally, the funnel plot technique was used to confirm the conclusions obtained by Rosenberg´s fail-safe number (Palmer, 1999) and Spearman´s rank correlation was used to formally test for publication bias (Begg & Mazumdar, 1994).

RESULTS

Among the studies examined that met the criteria selected for inclusion in the meta-analyses, only 51 studies were suitable (49 published studies, 2 unpublished datasets) (Appendix 2). However, not all these studies provided data for all the response variables studied. So, survival and size (i.e. length and mass) were analysed using higher number of publications and, as a consequence, data points (survival: 36 studies, 270 point samples; length: 7 studies, 111 point samples; weight: 19 studies, 187 point samples) than time to hatching (4 studies, 23 point samples), time to metamorphosis (9 studies, 37 point samples) and abnormality rate (5 studies, 39 point samples). In relation to the factorial meta-analysis of amphibian survival, needed data were obtained only from 13 studies (all of them published), which provided 48 data points.

Meta-analysis

Effects of pollutants on survival

Significant heterogeneity for survival was detected (Table 1). Overall, the exposure to pollutants had a negative effect on survival (Fig. 2). No significant differences between families or between developmental stages at the beginning of the experiment were recorded (Table 1). Nevertheless, Ranidae, Bufonidae, Ambystomatidae and Pipidae significantly reduced survival whereas the rest of families did not (Fig. 3, Appendix 3).

Significant differences were detected for the experimental venue and the kind of pollutant (Table 1). So, when individuals were exposed to pollution either in laboratory conditions or outdoor mesocosms and enclosures, the effect was negative. However, survival decline was more acute in the enclosures. Furthermore, with the exception of phosphorous compounds and heavy metals, the exposure to pollutants produced a significant negative effect, being such effect larger for road de-icers than for nitrogenous compounds (Fig. 3, Appendix 3).

Table 1. Heterogeinity statistics for each model in the survival, time to hatching and time to metamorphosis analyses. NA: not applicable; df: degrees of freedom; ^{BG}: between groups (refering to the variation in effect size explained by the model, Q_B). For clarity, the residual error heterogeneity (Q_W) corresponding to the different statistical models is not shown. With the exception of time to hatching and to metamorphosis (for all the models) and abnormalities (only for family and pollutant models), the residual error heterogeneity was significant, which imply that there is still heterogeneity among effect sizes not explained by the model (Rosenberg *et al.*, 2000).

	Survivorship (n = 270)			Time to hatching (n = 23)			Time to metamorphosis (n = 37)		
Statistical model	df	Q	P	df	Q	P	df	Q	P
Full model (no estructure)	**269**	**459.6927**	**0.0001**	22	22.4266	0.43469	36	42.0538	0.22524
Family ^{BG}	7	6.3538	0.49910	**1**	**17.7472**	**0.00003**	**3**	**9.0037**	**0.02924**
Developmental stage ^{BG}	1	0.6666	0.41423	NA	NA	NA	**1**	**4.4301**	**0.03531**
Experimental venue ^{BG}	**2**	**14.8828**	**0.00059**	NA	NA	NA	1	0.7926	0.37331
Pollutant ^{BG}	**5**	**15.1568**	**0.00971**	1	0.4231	0.51541	**3**	**8.9888**	**0.02944**
	Length (n = 111)			Weight (n = 187)			Abnormalities (n = 39)		
Statistical model	df	Q	P	df	Q	P	df	Q	P
Full model (no estructure)	**110**	**144.6966**	**0.01484**	**186**	**239.4115**	**0.00501**	**38**	**55.1173**	**0.03577**
Family ^{BG}	5	2.6392	0.75540	4	8.4054	0.07781	6	10.2068	0.11621
Developmental stage ^{BG}	2	0.9816	0.61213	**2**	**59.0227**	**0.0001**	1	1.4683	0.22561
Experimental venue ^{BG}	2	1.5748	0.45502	**3**	**18.1673**	**0.00041**	1	0.9308	0.33466
Pollutant ^{BG}	**2**	**6.7331**	**0.03451**	**3**	**12.6844**	**0.00537**	**3**	**19.1143**	**0.00026**

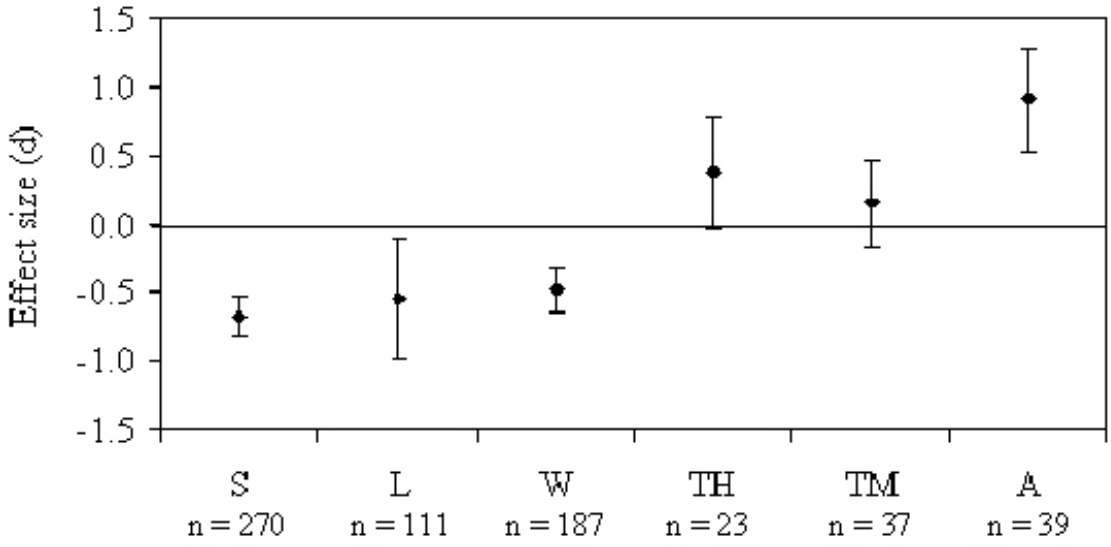


Fig. 2. Full models for the effect of pollutants on amphibian survival (S), length (L) and weight (W), time to hatching (TH), time to metamorphosis (TM) and abnormalities (A). The number of point samples used to calculate each mean is shown. The mean and 95% confidence interval is shown for each analysis. Confidence intervals that overlap the line at zero are not significantly different from zero.

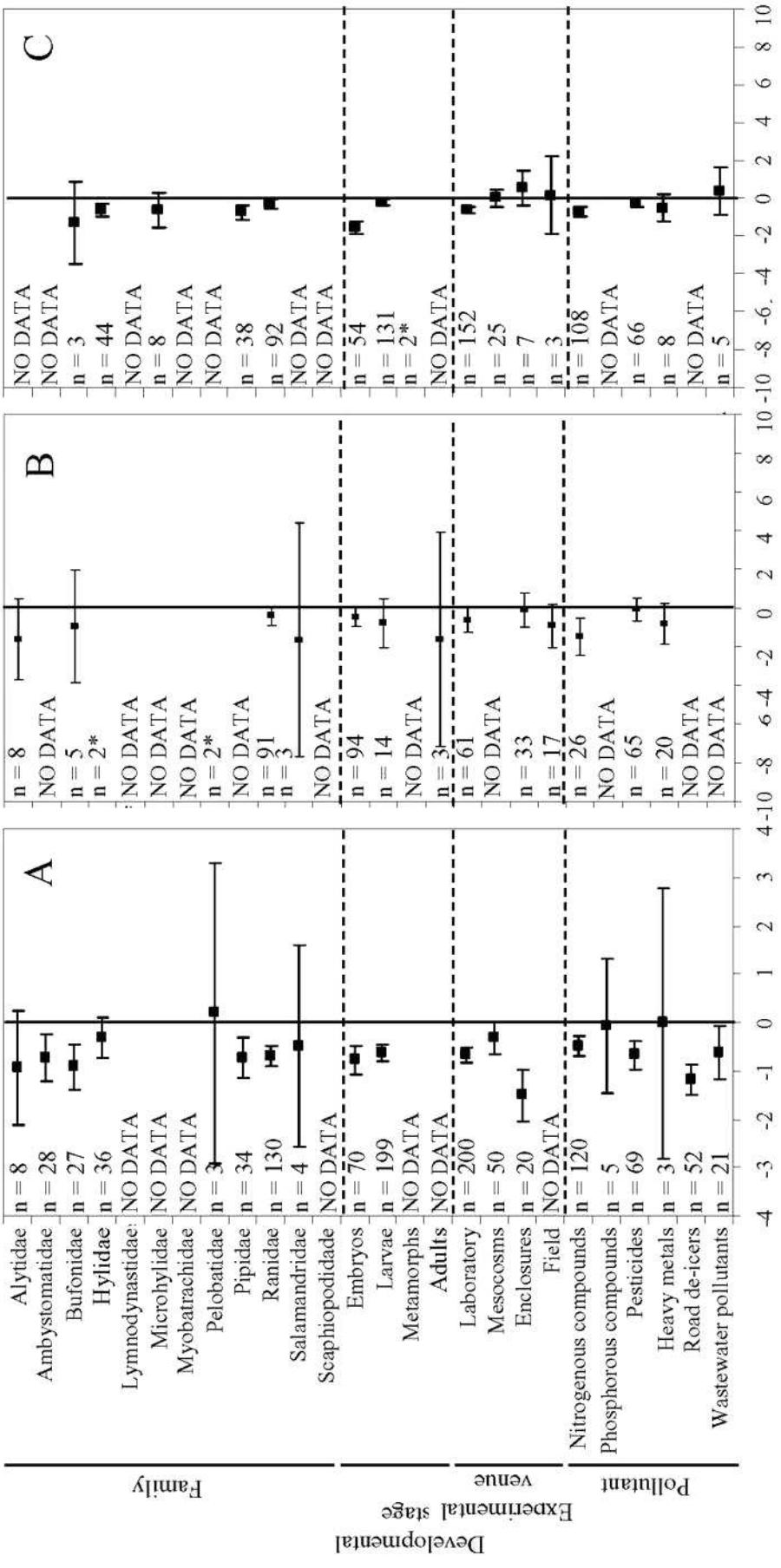


Fig. 3. Effect of pollutants on the categories considered for the *a priori* defined groups. A) survival; B) total length; C) weight. The number of point samples used to calculate each mean is shown. The mean and 95% confidence interval is shown for each analysis. Confidence intervals that overlap the line at zero are not significantly different from zero. Effect sizes within analyses were considered different from one another if their 95% confidence intervals did not overlap. Notice the different scales for each variable shown. * For clarity, statistics corresponding to categories showing small sampling size (n ≤2) are not shown in the graphic.

Effects of pollutants on size

The exposure to pollutants had a significally and moderate negative effect on length (Table 1, Fig. 2). Only significant differences among pollutants were detected. The exposure to nitrogenous compounds significantly reduced final length (Fig. 3, Appendix 3).

Final weight was significantly affected by the exposure to pollutants on overall, showing a moderate negative effect (Table 1, Fig. 2 and Appendix 3). Unlike length, significant differences among developmental stages, experimental venue and pollutant were found and a marginally family effect was revealed, showing only Hylidae, Pipidae and Ranidae a negative effect size, being smaller for Ranidae (Table 1, Fig. 3 and Appendix 3). Therefore, when the exposure to pollution began at embryonic or larval stages, the experiment ran in laboratory conditions and the pollutant consisted of nitrogenous compounds, a significant reduction in final mass was detected.

Effects of pollutants on development

The analysis of both time to hatching and time to metamorphosis revealed the lack of significant heterogeneity (Table 1). Nevertheless, significant differences among families were detected for time to hatching (Table 1). Only Ambystomatidae showed significant delay in hatching time under pollutants (Fig. 4, Appendix 3). No significant differences in both traits were detected for the rest of the previouly defined groups (Table 1, Fig. 4, Appendix 3).

Abnormalities

On overall, abnormality rate increased as a consequence of the exposure to pollutants (Table 1; Fig. 2, Appendix 3). Significant differences were detected among pollutants,

being the largest effect for wastewater contaminants and, to a lesser extent, for nitrogenous compounds (Fig. 4, Appendix 3). Significant differences among the rest of categories were not observed (Table 1). Nevertheless, increased abnormalities were recorded for both the families Ambystomatidae and Ranidae and for embryonic developmental stages and laboratory conditions (Fig. 4, Appendix 3).

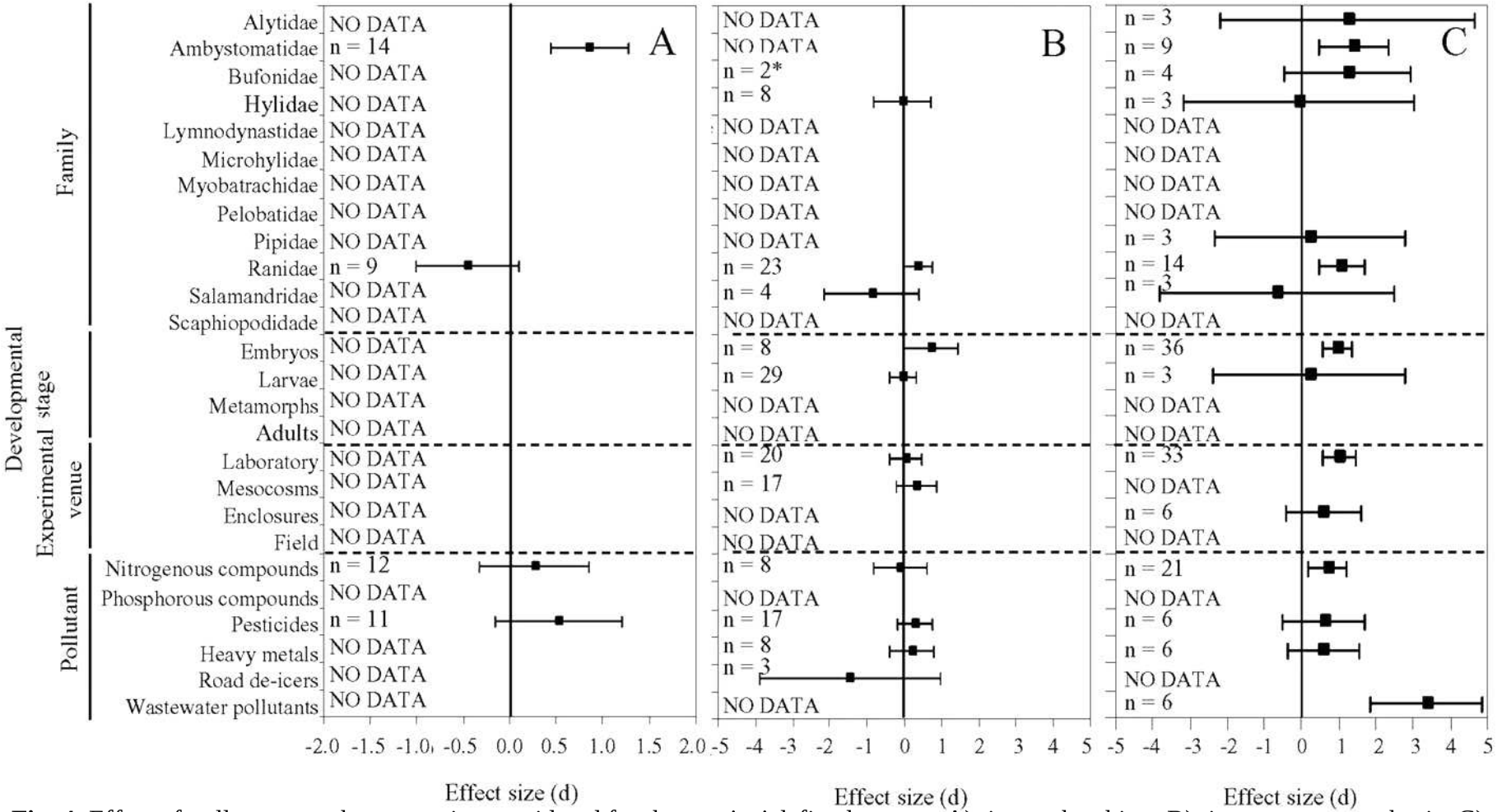


Fig. 4. Effect of pollutants on the categories considered for the *a priori* defined groups. A) time to hatching; B) time to metamorphosis; C) abnormalities. The number of point samples used to calculate each mean is shown. The mean and 95% confidence interval is shown for each analysis. Confidence intervals that overlap the line at zero are not significantly different from zero. Effect sizes within analyses were considered different from one another if their 95% confidence intervals did not overlap. Notice the different scales for each variable shown. * For clarity, statistics corresponding to categories showing small sampling size (n ≤2) are not shown in the graphic.

Factorial meta-analysis

Significant heterogeneity was detected for all the parameters computed ($P < 0.03$ in all cases), with the exception of the di ($Q_{47} = 48.073$; $P = 0.429$) and dp, ns ($Q_{47} = 55.003$; $P = 0.192$). The average overall effects on survival of the exposure to nitrogenous compounds, pesticides, and wastewater pollutants (FGSF, dp) were significantly milder than the effect of the second group of stressors (competitors, pH, predators, UV radiation, other wastewater pollutants and mold) (SGSF, ds) (between groups heterogeneity: $Q_1 = 10.634$; $P = 0.001$) (Fig. 5). The exposure to competitors, pH, predators, UV radiation, other wastewater pollutants and mold in the presence (ds, p) or absence of nitrogenous compounds, pesticides, and wastewater pollutants (dnp, s) produced a reduction in survival but no significant differences were found between both groups (between groups heterogeneity: $Q_1 = 0.071$; $P = 0.791$) (Fig. 5). The average effect of the exposure to nitrogenous compounds, pesticides, and wastewater pollutants when the second group of stressors was absent was low-moderately negative (dp, ns = -0.3032, 95% CI= -0.5655 - -0.0409, Fig. 5) whereas when it was present (dp, s) no significant effects on survival were observed (Fig. 5). However, no significant differences between both effect sizes were detected (between groups heterogeneity: $Q_1 = 0{,}408$; $P = 0.523$). The interaction effect size did not differed from 0 (di = 0.1838, 95% CI = -0.2811 – 0.6487; Fig. 5).

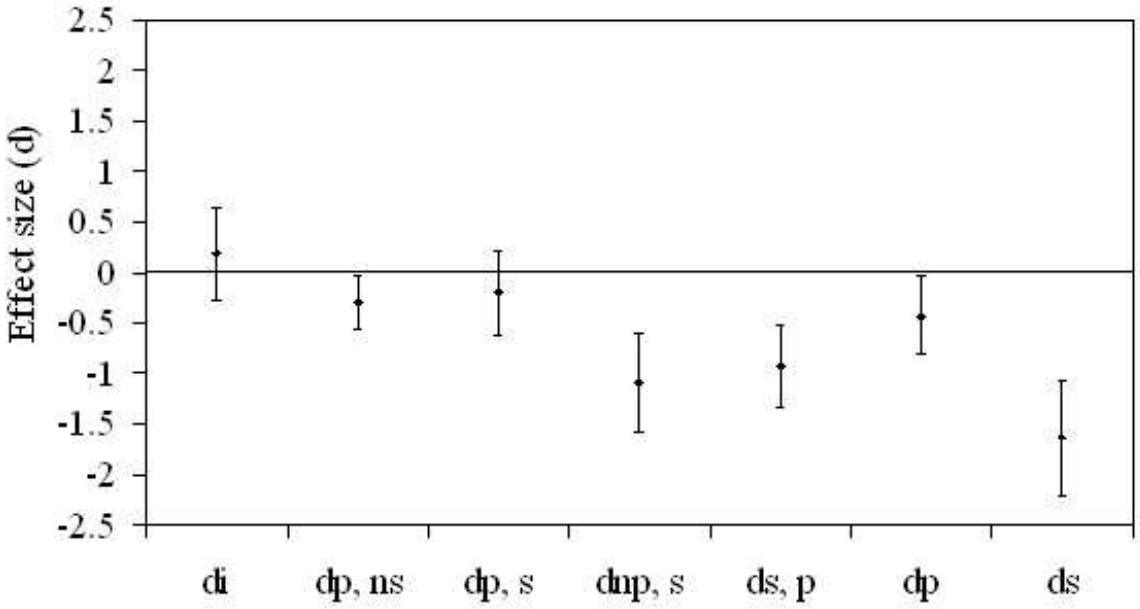


Fig. 5. Full models for the parameters calculated for the factorial meta-analysis of amphibian survival (n = 48). The mean and 95% confidence interval is shown for each analysis. Confidence intervals that overlap the line at zero are not significantly different from zero. di: average interaction effect size between the exposure to FGSF and to SGSF; dp, ns: average effect sizes of exposure to FGSF in the absence of SGSF; dp, s: average effect sizes of exposure to FGSF in the presence of SGSF; dnp, s: average effect sizes of exposure to SGSF in the absence of FGSF; ds, p: average effect sizes of exposure to SGSF in the presence of FGSF; dp: average overall effect sizes of exposure to FGSF; ds: average overall effect sizes of exposure to SGSF.

Phylogenetic comparative analysis

The TFSI tests showed significant phylogenetic autocorrelation among the tip data for the effect size (i.e. phenotypic plasticity induced by pollutants) in the case of time to hatching (Table 2), not existing indication of phylogenetic signal for the rest of the response variables studied (Table 2).

Considering the factorial meta-analysis performed on survival, TFSI revealed the existence of significant phylogenetic autocorrelation for the effect size for the exposure to FGSF when SGSF was absent (dp, ns) and for the average effect of the exposure to FGSF (dp) (Table 2).

Table 2. Results of the tests for serial independence (TFSI) for the response variables analysed. Those response variables for which significant phylogenetic autocorrelation was observed appear in bold.

Response variable	Mean *C*-statistic
Survival	-0.1624
Time to metamorphosis	-0.1108
Time to hatching	**0.4613**
Length	0.06840
Mass	-0.1439
Abnormalities	-0.1010
Survival-Factorial meta-analysis	
dp, ns	**0.2435**
dp, s	0.1435
ds, p	-0.03967
dnp, s	0.04883
dp	**0.1982**
ds	-0.003231
di	0.02388

Publication bias

The weighted Rosenberg´s failsafe number was large for survival (2673.2) and weight (818.5), whereas it was smaller for abnormalities (12.3) and length (5.0). In the case of time to hatching and time to metamorphosis, for which effect sizes did not significantly differ from 0, Rosenberg´s failsafe number equals to 0. As regards the factorial meta-analysis of survival, Rosenberg´s failsafe number was low for all the effect sizes analysed, whith the exception of those corresponding to the effects of stressing factors on amphibians (ds, p and dnp, s) (Table 3).

Spearman rank correlation test was not significant for time to metamorphosis (R= 0.013; P= 0.939), time to hatching (R= -0.087; P= 0.693), abnormalities (R= 0.235; P= 0.151) and survival (R= 0.113; P= 0.0627). In the case of length (R= -0.225; P= 0.0177) and weight (R= 0.365; P= 0.0001), significant results were obtained. The factorial meta-analysis of survival revealed that significant Spearman rank correlation tests were obtained for the average effect of the exposure to SGSF (ds) and for the effect of SGSF when FGSF was absent (dnp, s) (Table 3). All these results suggest that, except for length, weight, and the factorial meta-analysis effect sizes ds and dnp, s, there were not publication bias. However, this interpretation was not supported by the skewed funnel plots of effect size versus sample size observed for time to hatching, abnormalities and all the parameters computed to perform the factorial meta-analysis (Appendix 4).

Table 3. Rosenberg´s failsafe numbers and results of the Spearman rank correlation tests for the parameters computed to perform the factorial meta-analysis on amphibian survival (n = 48 in all cases).

		Spearman rank correlation	
Parameter	Rosenberg´s failsafe number	Rs	P
di	0.0000	0.238	0.10297
dp, ns	12.9468	-0.076	0.60951
dp, s	0.0000	0.241	0.09838
dnp, s	68.1542	-0.475	0.00064
ds, p	84.1853	-0.251	0.08529
dp	1.8422	0.162	0.26983
ds	38.2759	-0.452	0.00125

DISCUSSION

The results obtained revealed that the exposure to pollutants had, on overall, a moderate-large negative effect, both lethal and sublethal, on amphibians. This fact supports the conclusions shown in traditional reviews on the effect of pollutants on these vertebrates (Cowman & Mazanti, 2000; Linder & Grillitsch, 2000; Sparling, 2000; Camargo *et al.*, 2005; Relyea & Hoverman, 2006; Marco & Ortiz-Santaliestra, in press), and agrees with the hypothesis describing pollution as one of the major threats that these vertebrates are facing currently (e.g. Beebee & Griffiths, 2005). Moreover, the comparisons made showed patterns that previous reviews were unable to describe. The impact of pollution varied among response variables, being survival and abnormality rates largely affected and size moderately influenced, whereas no significant effect was observed for developmental rates both time to hatching and to metamorphosis. Although individual effects may not be literally translated into populational effects (Schmidt, 2004), understanding how individuals face multiple stressors impact on natural populations is essential (Sih *et al.*, 2004), since individual traits such as larval mortality may have deletereous effects at population level (Gamradt & Kats, 1996;

Vredenburg, 2004). Thus, pollution may lead to population decline directly by reducing individual survival and indirectly by affecting other sublethal fitness related traits, such as size and abnormality rates, which are correlated with parameters as important to juvenile and adult fitness as size at metamorphosis, early juvenile survival, adult fecundity, survival and size at first reproduction (Berven & Gill, 1983; Smith, 1987; Semlitsch, *et al.*, 1988; Reques & Tejedo, 1997; Altwegg & Reyer, 2003). Nevertheless, the results obtained suggest that the duration of embryonic and larval development is not affected by pollution. It suggests that other strong selective factors such as pond dessication are unlikely to indirectly affect larval survival as a consequence of longer stays in temporary aquatic environments.

Survival

On overall, embryonic and larval amphibian survival was severely affected by pollutants. This negative effect may be due to physiological alterations, such as increased methaemoglobin concentrations, modification of enzyme activities and even DNA damage (e.g. Huey & Beitinger, 1980; Ralph & Petras, 1997; Widder & Bidwell, 2006). Moreover, the significant larger impact of road de-icers observed in relation to nitrogenous compounds may be due to the interaction between de-icers and water conductivity, since most of the publications dealing with the effect of these pollutants focused on the effect of water conductivity rather on the effect of road de-icers by themselves (e.g. Karraker, 2007; Karraker *et al.*, 2008). The results obtained support the hypothesis suggesting a lack of effect of phosphorous compounds on amphibians (Smith, 2007, but see Hamer *et al.*, 2004). Previous studied have stated that amphibians inhabiting polluted habitats may be threatened by the exposure to heavy metals (Linder & Grillitsch, 2000). However, the results obtained do not support this hypothesis,

although this may be due to small sample size and eventual error type II. Moreover, further examination of the studies included in our meta-analysis revealed that the lack of effect of heavy metals on amphibian survival may be mediated by the elevated mortality in control treatment (Chen *et al.*, 2006), increasing also error type II. Thus, further research is needed to verify the results obtained in relation to the effect of heavy metals on survival.

The exposure to pollutants in enclosure experiments and, to a lesser extent, in laboratory conditions significantly reduced embryonic and larval amphibian survival. The exposure to the combination of pollutants and other stressing factors present in the field may affect amphibians more severely than when they act isolated in the laboratory (e.g. Hatch & Blaustein, 2000; Boone *et al.*, 2005; Macías *et al.*, 2007; Egea-Serrano *et al.*, 2009). Therefore, the larger negative effect observed for enclosure experiments can be explained due to the additive and synergistic interactions with other stressors which would exacerbate the effects obtained in laboratory conditions (this thesis, see discrepancies between chaper 4 and 7).

Evidences on ontogenic and interspecific variation in relation to vulnerability to pollutants have been previously found (e.g. Marco *et al.*, 1999; Bridges, 2000; Bridges & Semlitsch, 2000; Greulich & Pflugmacher, 2003; Ortiz-Santaliestra *et al.*, 2006; Shinn *et al.*, 2008; Snodgrass *et al.*, 2008). Therefore, we would have expected significant differences in survival among developmental stages and species. However, although individual survival in polluted environments was lower than in control treatments for all the developmental stages analysed, no differences between stages were detected. Aspects such as the presence of gelatinous matrix and complete tissue and organ differentiation have been argued to explain ontogenic differences in amphibian tolerance to pollutants (Berrill *et al.*, 1998; Pauli *et al.*, 1999; Ortiz-

Santaliestra *et al.*, 2006). However, present results would evidence that on overall these characteristics are not a reliable protection against pollution. In addition, it is likely that the impact of pollutants on embryos is overstimated, since the duration of pollutant exposition is significantly longer for them than when the experiment began at larval stages (mean ± 1 SE; embryos: 750.95 h ± 107.37 h, n = 76; larvae: 446.10 h ± 34.11 h, n = 286; $F_{1,360}$ = 12.514; P = 0.0001). Finally, the lack of interspecific differences in relation to the effect of pollution on survival disagrees with the results shown in previous publications in relation to pollutants such as pesticides or nitrogenous compounds (e.g. Marco *et al.*, 1999; Shinn *et al.*, 2008; Snodgrass *et al.*, 2008). This result would point out that pollution is an universal threat to amphibians, since there are not families more tolerant than others, fact that would agree with the lack of phylogenetic autocorrelation detected.

Size

The overall negative effect of pollutants on size may be both direct, mediated by reduced foraging efficiency or by the physiological stress due to detoxification pathways (Wright & Wrigth, 1996; Egea-Serrano *et al.*, 2009), and indirect, since pollutants may help algal growth (Boone *et al.*, 2007; Egea-Serrano *et al.*, chapter 7 in the present thesis) and, consequently, enhance eutrophication processes. No significant differences between amphibian families were detected, which would support the lack of phylogenetic autocorrelation detected for this trait. However, the results obtained revealed that the magnitude of the effect of pollutants on size varied between developmental stage, experimental venue and type of pollutant. Pollutant effect on mass was significantly more negative when the exposure began at embryonic stages than when it began at larval stages. Such effect may be mediated by the longer exposure to

pollutants when experiments began at embryonic stages (see above) or may be a consequence of either the incomplete tissue and organ differentiation of embryos being more unprotected against pollutants (Herkovits & Fernández, 1978), or to higher vulnerability to osmoregulatory alteration (McDiarmid & Altig, 1999) or even to the interaction between pollutants and some components of the jelly coat, which would make it more toxic to embryos (Marquis *et al.*, 2006a). Although the alteration of density-dependent traits, such as survival, at early life stages may have small impact on amphibian populations and communities that at later stages (Vonesh & De la Cruz, 2002), the negative effect on mass observed when embryos were exposed to pollutant may involve detrimental effects on individual fitness and, eventually population viability, since size is correlated with adult fitness traits such as early juvenile survival, fecundity, survival and size at first reproduction (Berven & Gill, 1983; Smith, 1987; Semlitsch, *et al.*, 1988; Reques & Tejedo, 1997; Altwegg & Reyer, 2003). Moreover, reduced size may make amphibians more vulnerable to gape-size predators (Semlitsch & Gibbons, 1988) and may reduced competitive abilities or increase larval development duration (Snodgrass *et al.*, 2004).

Amphibians exposed in laboratory conditions to pollutants significantly reduced their size when compared with mesocosm or enclosures experiments. Pollution may affect amphibians directly, modifying fitness-related traits, or indirectly, altering food web (e.g. Watt & Oldham, 1995; Boone *et al.*, 2007). The addition to water bodies of fertilizers or pesticides may help algal growth (Boone *et al.*, 2007; Egea-Serrano *et al.*, chapter 7 in the present thesis), increasing food availability for most anuran larvae. Because of the strictly controlled environmental conditions in laboratory experiments, it is unlikely a significant increase of algal biomass in experimental beakers. Thus, the negative effect associated with laboratory conditions may be due to the direct impact of

pollutants, whereas surviving amphibians in enclosure and mesocosm experiments were able to take advantage of the lack of food limitation and therefore pollutants become a facilitation and not stressful factor such it has been suggested with other stressors such as predation risk (Peacor & Werner, 2000).

Nitrogenous compounds affected more negatively amphibian size than pesticides. Previous studies have described similar effects of pesticides and nitrogenous compounds on amphibian mass (Boone *et al.*, 2005, 2007; Boone & Bridges-Britton, 2006). However, the significant differences observed do not support the results reported in original studies. Nitrogenous compounds may increase water salinity (e.g. Egea-Serrano *et al.*, chapters 4 and 7 in the present thesis) and lead to oxygen depletion because of eutrophication processes (see review Camargo & Alonso, 2006). These additional stressing effects may have exacerbated amphibian response to direct toxicity of nitrogenous compounds (e.g. Ortiz-Santaliestra, 2008), which would contribute to explain the results obtained.

Development

Although evidences on the significant effect of pollutants on time to hatching have been published (e.g. Ingermann, 1997; Rohr *et al.*, 2003, 2004; Griffis-Kyle, 2007), many studies have reported no impact (Berrill *et al.*, 1994, 1998; Berrill & Bertram, 1997; Greulich & Pflugmacher, 2003; Pauli *et al.*, 1999; Griffis-Kyle, 2007). Both the observed lack of overall effect and of significant heterogeneity between types of pollutants would agree with this last scenario. Assuming a lack of pollutant effect may be explained by considering the protecting role of embryonic jelly coat (Räsänen *et al.*, 2003; Marquis *et al.*, 2006b; Edginton *et al.*, 2007) or the incomplete embryonic organ development (Hecnar, 1995). However, the suggested protecting mechanisms were not

effective for other studied variables, since they were significantly affected when the exposure to pollutants began at embryonic stages. This fact would suggest that pathways involved in embryonic development do not affect other traits, as it has been described for the lack of effect of decreased growth on larval development (Bridges, 2000).

Significant heterogeneity among families was observed in relation to time to hatching. Ambystomatids delayed their embryonic development, whereas ranids were unaffected. This result is also supported by the significant phylogenetic autocorrelation detected and agrees with the information reported in original studies (Griffis-Kyle, 2007). This fact would suggest that pollutants severely affect the rate of cellular division in the case of ambystomatids. The extended embryonic period caused by the exposure to pollutants and other stressing factors, such as conspecific cues, is not associated to a more developed stage or to larger size (Griffis-Kyle, 2007; Mandrillon & Saglio, 2008). Thus, the oxygen consumption efficiency is lower (Griffis-Kyle, 2007), which may negatively affect the survival of the newly hatched individuals. Moreover, the delayed embryonic develoment exposes ambystomatids to an increased risk of mortality as a consequence of water body dessication, one of the major threats described for some species of ambystomatids (Petranka, 1998).

The lack of effect of pollutants on time to metamophosis, together with the absence both of differences among the categories for the *a priori* defined groups and phylogenetic autocorrelation, would point out that the impact of pollutants on amphibian development is not significant, regardless the type of compound. This would suggest that amphibians may be not indirectly threatened by other stressors (e.g. pond dessication) because of longer stays in aquatic environments.

Abnormalities

The exposure to wastewater pollutants significantly increased, on overall, the incidence of abnormalities likely due to the alteration of those enzymes involved in development or to DNA damage (Dunson & Connell, 1982; Ralph & Petras, 1997). Physical abnormalities are correlated with reduced speed and anomalous movements (Laposata & Dunson, 1998). Since foraging abilities and vulnerability to predators are associated with locomotor abilities (e.g. Watkins, 1996; Dayton & Fitzgerald, 2001), and abnormal movement may imply increased metabolic costs (Rowe *et al.*, 2002), the observed effects of pollutants on abnormality rate may severely affect individual fitness. In spite of the lack of significant differences for the *a priori* defined categories and the lack of phylogenetic autocorrelation, significant heterogeneity among types of pollutants was detected and enhanced abnormalities occurred under wastewater pollutants. The only wastewater pollutant considered in our meta-analysis was boron cation. Laposata & Dunson (1998) suggested that cations may affect more severely the hatching enzyme responsible for enlarging the perivitelline membrane surrounding the embryo (Dunson & Connell, 1982) than anions (e.g. nitrate), increasing so the incidence of malformations. This hypothesis would explain the higher effect of wastewater pollutants observed. Together with these pollutants, nitrogenous compounds increased the incidence of malformations. Therefore, a negative impact of these compounds on amphibian fitness would be expected, since individual malformations are related to reduced speed and displacement type (Laposata & Dunson, 1998) and, consequently to increased susceptibility to predators (e.g. Watkins, 1996).

The effect of multiple stressors. Do pollutants act synergistically?

Evidences on the increased impact of the combination of stressors have been published (e.g. Hatch & Blaustein, 2000; Boone *et al.*, 2005; Hayes *et al.*, 2006; Macías *et al.*, 2007; Egea-Serrano *et al.*, 2009, but see Boone & Bridges-Britton, 2006). However, the results on survival provided by the factorial meta-analysis do not support the additive or synergistic hypothesis. The larger overall negative effect of the second group of stressors (i.e. competitors, pH, predators, UV radiation, other wastewater pollutants and mold) in relation to the effect of nitrogenous, pesticides, and wastewater pollutants evidences that the exposure to a wide range of stressors, both biotic and abiotic, is more harmful to amphibian survival than the exposure to full pollutant stressors. Nevertheless, these results need to be considered cautiously because publication bias exists regarding the factorial meta-analysis, as revealed by the skewed funnel plots, reduced Rosenberg´s failsafe numbers and significant Spearman rank correlations.

The overall interpretation of the results provided by funnel plots, Spearman rank correlations and Rosenberg´s failsafe numbers revealed that for those response variables for which a significant effect of pollutant was observed no publication bias exists. Therefore, only for time to hatching and time to metamorphosis publication bias was detected. According to the publication bias hypothesis, if selective reporting exists, studies showing significant effects would be mainly published (Rosenberg *et al.*, 2000). Consequently, the publication bias detected for time to hatching and time to metamorphosis would suggest that for these variables only the most relevant results have been published. Even so, no significant effect size was recorded, which would reinforce our conclusion on the overall lack of effect of pollutants on amphibian development.

Conclusions

As regards to the effects of pollutants on amphibians, the present study is, to our knowledge, the first attempt to quantitative assess the impact of chemical pollutants on amphibians using rigorous meta-analysis statistical procedures. The results obtained evidence that pollution is a major threat to amphibians, although they preliminary suggest that the exposure to a great variety of biotic and abiotic stressors play a larger effect on survival. In spite of this overall negative effect, our results described the great variation existing in relation to the effect of pollutants among types of compounds, experimental venue, developmental stage and even phylogeny. This fact emphasizes the relevance of considering this heterogeneity when assessing the actual impact of pollution on amphibians.

The results obtained highlight the negative impact of pollution on amphibians. However, it is noticeable that the response variables considered in the present study are only a sample of the many endpoints which can be affected by pollution. Since other sublethal impacts may occurr (e.g. Hayes *et al.*, 2006), further studies on other health traits are needed. Moreover, the examination of the interaction among several stressors is necessary to understand how pollutants and other stressing factors act when they are combined at both individual and community level, since analysing the impact of only one type of compound may understimate the impact of pollutants on amphibians (Hayes *et al.*, 2006).

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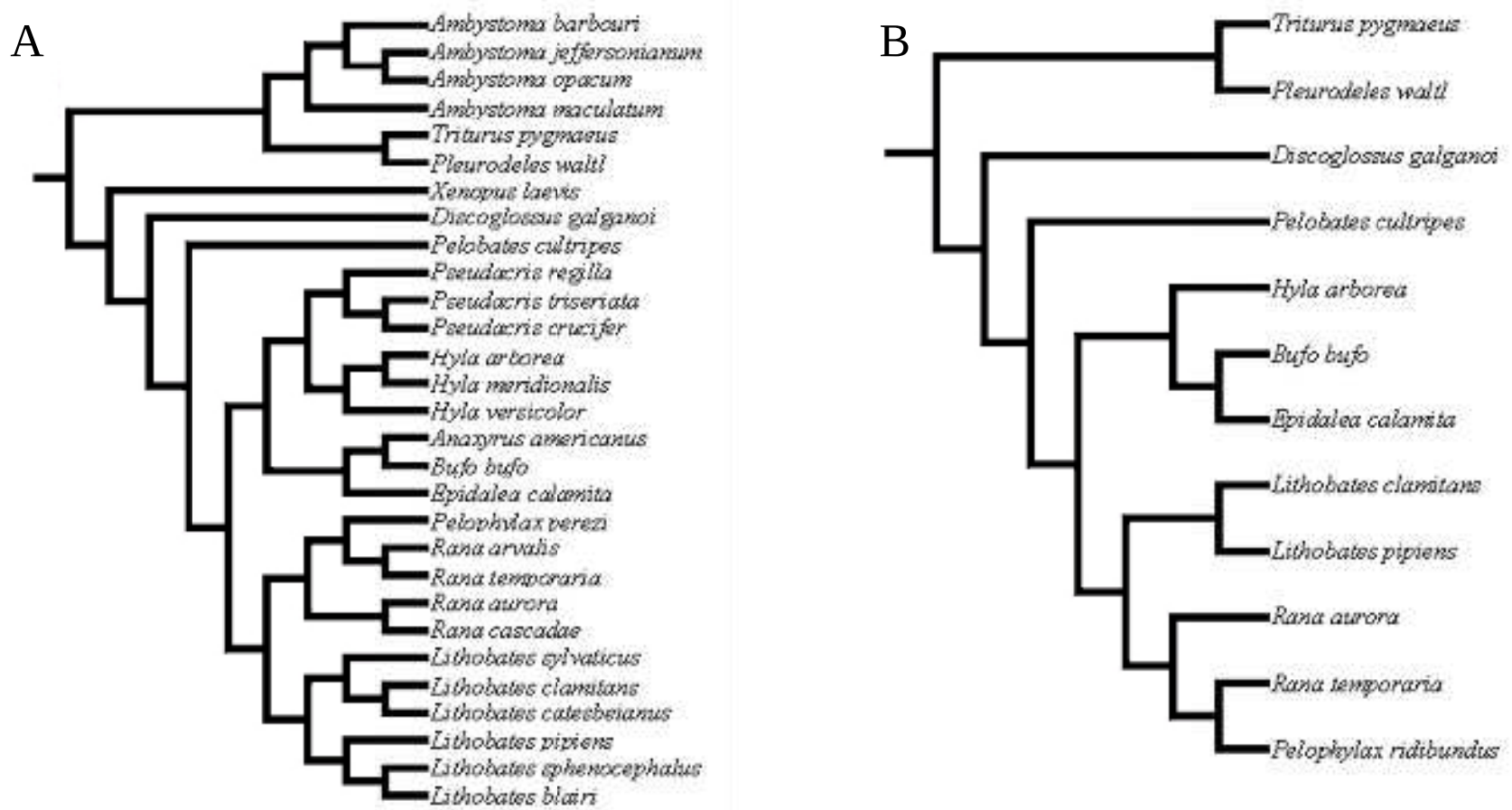
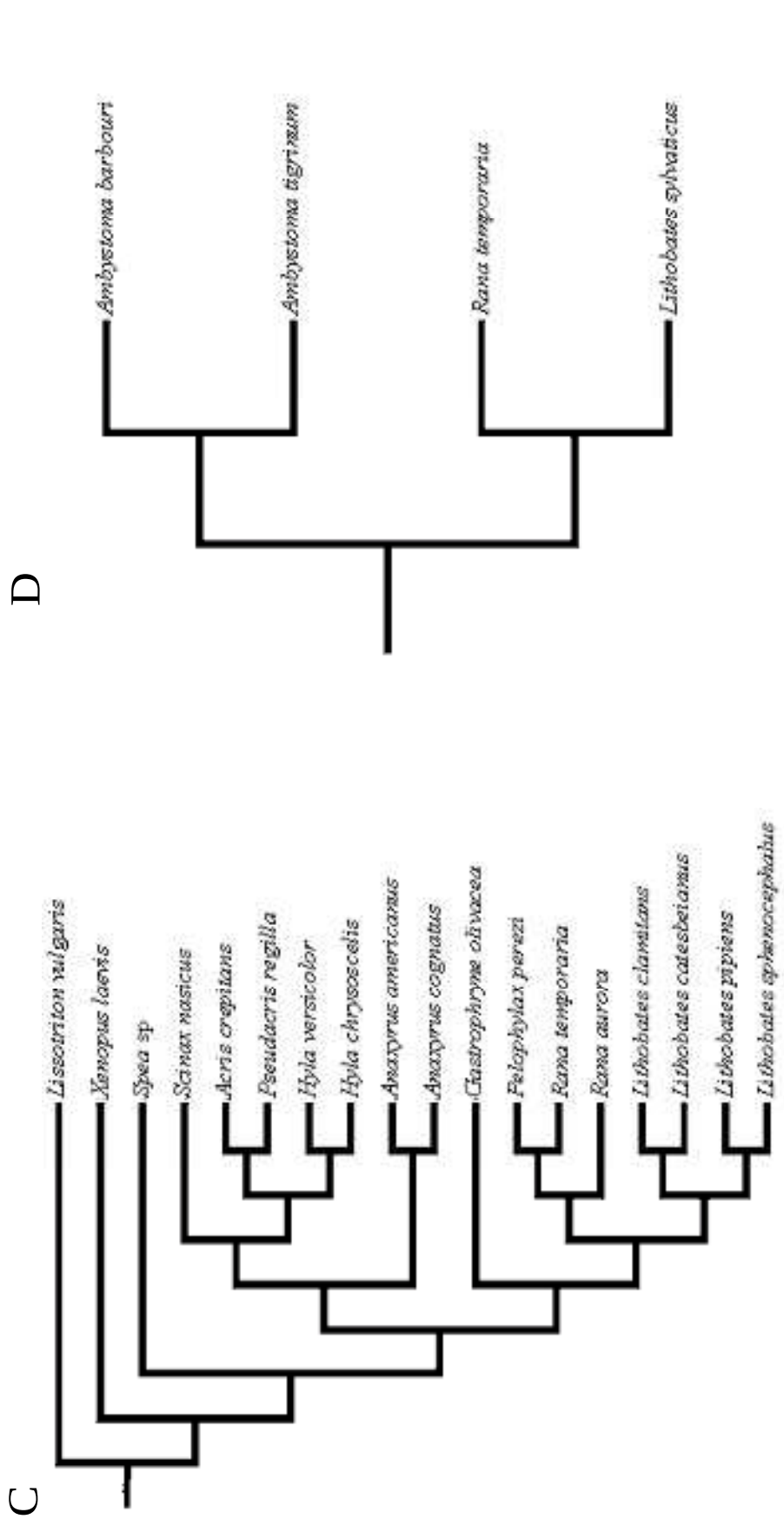
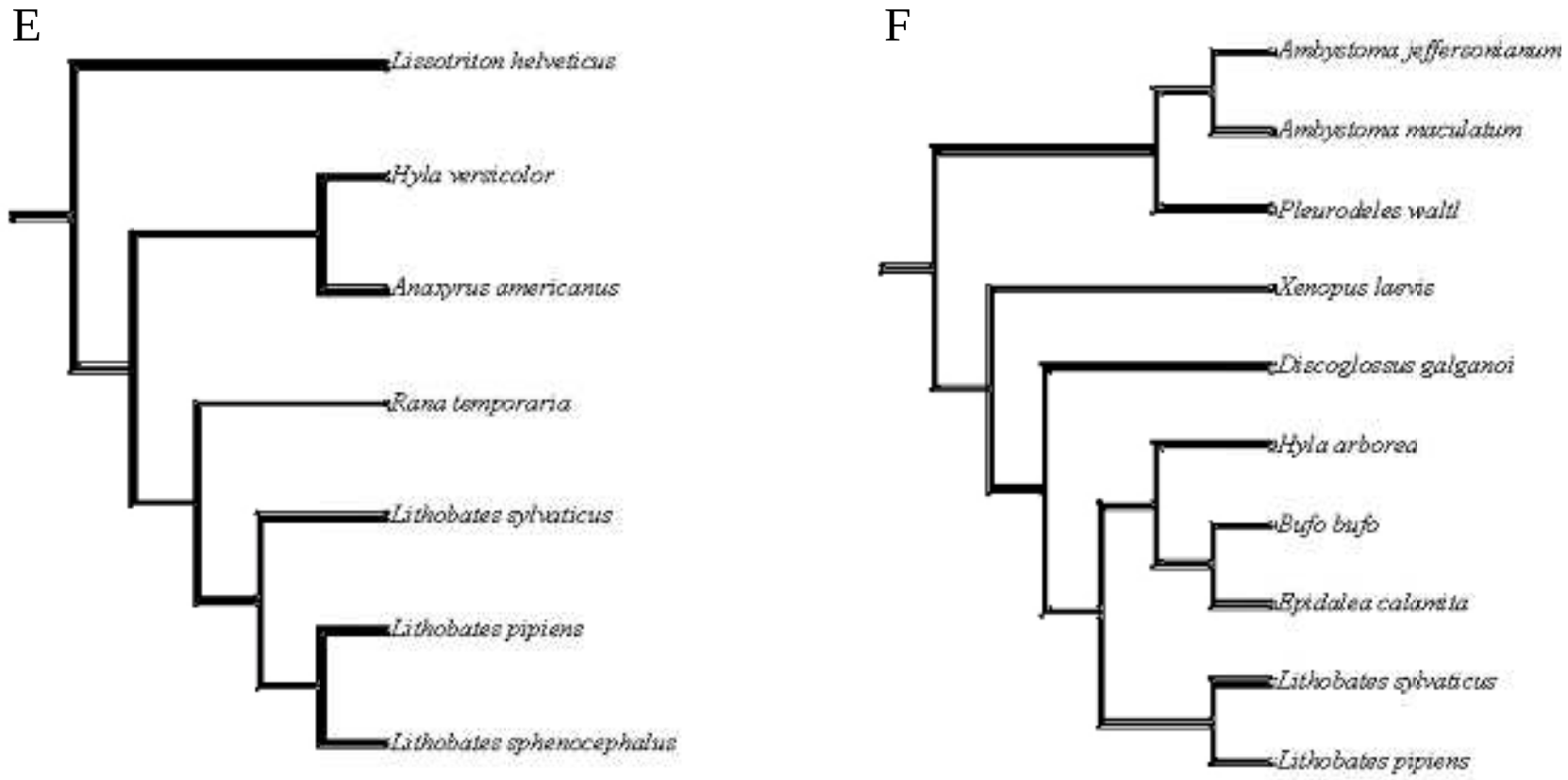
APPENDICES



Appendix 1. Phylogenetic relationships among amphibian families used to perform test for serial independence (TFSI). A) Survival (simple meta-analysis); B) Total length; C) Weight; D) Time to metamorphosis; E) Time to metamorphosis; F) Abnormalities; G) Survival (factorial meta-analysis).



Appendix 1 (continued). Phylogenetic relationships among amphibian families used to perform test for serial independence (TFSI). A) Survival (simple meta-analysis); B) Total length; C) Weight; D) Time to metamorphosis; E) Time to metamorphosis; F) Abnormalities; G) Survival (factorial meta-analysis).



Appendix 1 (continued). Phylogenetic relationships among amphibian families used to perform test for serial independence (TFSI). A) Survival (simple meta-analysis); B) Total length; C) Weight; D) Time to metamorphosis; E) Time to metamorphosis; F) Abnormalities; G) Survival (factorial meta-analysis).

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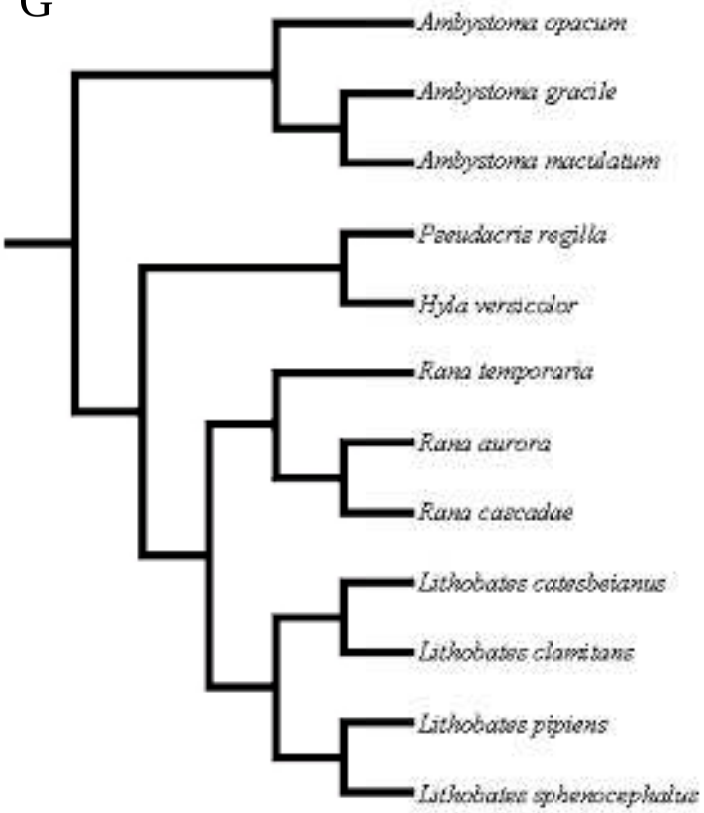

Appendix 1 (continued). Phylogenetic relationships among amphibian families used to perform test for serial independence (TFSI). A) Survival (simple meta-analysis); B) Total length; C) Weight; D) Time to metamorphosis; E) Time to metamorphosis; F) Abnormalities; G) Survival (factorial meta-analysis).

APPENDIX 2. References and unpublished data bases that fitted the criteria selected for inclusion in the meta-analyses of the response variables analysed in the present study. Superscripts refer to the references used to elaborate the data base for eah response variable analysed. 1: Survival (simple meta-analysis); 2: Survival (factorial meta-analysis); 3: time to hatching; 4: time to metamorphosis; 5: total length; 6: mass; 7: abnormalities.

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APPENDIX 3. Effect of pollutants on amphibians for each category considered in the *a priori* defined groups for the present study. ND: no data.

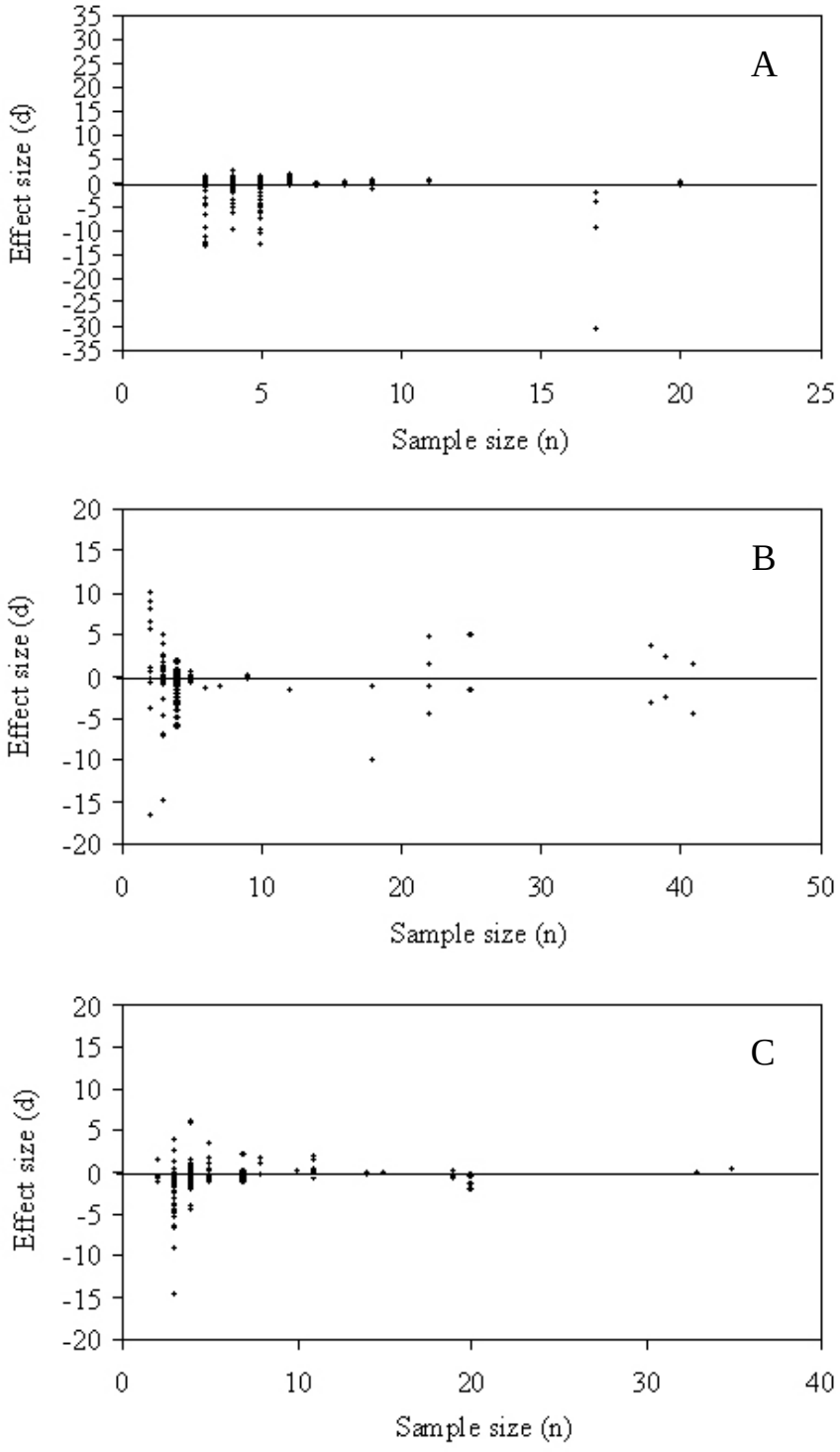
		Survival					Length				
			95% CI		BiasCI			95% CI		BiasCI	
Group	**Categories**	**E**	**Inferior**	**Superior**	**Inferior**	**Superior**	**E**	**Inferior**	**Superior**	**Inferior**	**Superior**
Family	Alytidae	-0.9524	-2.1320	0.2272	-2.2533	-0.3555	-1.6253	-3.7274	0.4768	-3.4364	-0.4557
	Ambystomatidae	-0.7344	-1.2174	-0.2513	-1.3941	-0.1872	ND	ND	ND	ND	ND
	Bufonidae	-0.9197	-1.3888	-0.4507	-1.3821	-0.5690	-0.9458	-3.8478	1.9561	-3.2417	-0.1009
	Hylidae	-0.3086	-0.7295	0.1124	-0.6860	0.0361	-0.7549	-21.6741	20.1644	-1.0116	-0.5011
	Lymnodynastidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Microhylidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Myobatrachidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Pelobatidae	0.1963	-2.9172	3.3097	0.1136	0.2792	-0.5334	-21.4006	20.3337	-0.6974	-0.3704
	Pipidae	-0.7295	-1.1307	-0.3283	-0.8975	-0.5595	ND	ND	ND	ND	ND
	Ranidae	-0.7080	-0.9164	-0.4996	-0.9369	-0.5220	-0.3933	-0.8861	0.0995	-0.9338	0.0772
	Salamandridae	-0.4838	-2.5720	1.6044	-1.9463	1.5785	-1.6533	-7.6827	4.3761	-5.8005	-0.0826
	Scaphiopodidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Developmental stage	Embryos	-0.7741	-1.0717	-0.4764	-1.1850	-0.4179	-0.4576	-0.9473	0.0321	-0.8664	-0.0931
	Larvae	-0.6344	-0.7995	-0.4693	-0.7826	-0.5063	-0.7813	-2.0474	0.4848	-3.0591	0.949
	Metamorphic	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Adults	ND	ND	ND	ND	ND	-1.6144	-7.1377	3.909	-1.9224	-1.4301
Experimental venue	Laboratory	-0.6744	-0.8400	-0.5088	-0.8137	-0.5547	-0.6248	-1.2271	-0.0225	-1.067	-0.2794
	Mesocosms	-0.3211	-0.6533	0.0110	-0.6140	-0.0289	ND	ND	ND	ND	ND
	Enclosures	-1.5124	-2.0585	-0.9663	-2.4682	-0.8335	-0.1128	-0.9939	0.7683	-0.9163	0.8758
	Field	ND	ND	ND	ND	ND	-0.9253	-2.0582	0.2076	-2.6591	0.8348
Pollutant	Nitrogenous compounds	-0.4972	-0.7105	-0.2839	-0.6844	-0.3478	-1.4682	-2.435	-0.5014	-2.2801	-0.8153
	Phosphorous compounds	-0.0591	-1.4458	1.3276	-0.3410	0.2346	ND	ND	ND	ND	ND
	Pesticides	-0.6753	-0.9654	-0.3853	-1.0196	-0.3942	-0.0682	-0.6623	0.526	-0.5306	0.4352
	Heavy metals	-0.0115	-2.8035	2.7805	-0.9386	0.5036	-0.8128	-1.8452	0.2195	-2.4047	0.4782
	Road de-icers	-1.1822	-1.5070	-0.8575	-1.5789	-0.8810	ND	ND	ND	ND	ND
	Wastewater contaminants	-0.6255	-1.1675	-0.0835	-1.1590	-0.2575	ND	ND	ND	ND	ND

APPENDIX 3 (continued). Effect of pollutants on amphibians for each category considered in the *a priori* defined groups for the present study. ND: no data.

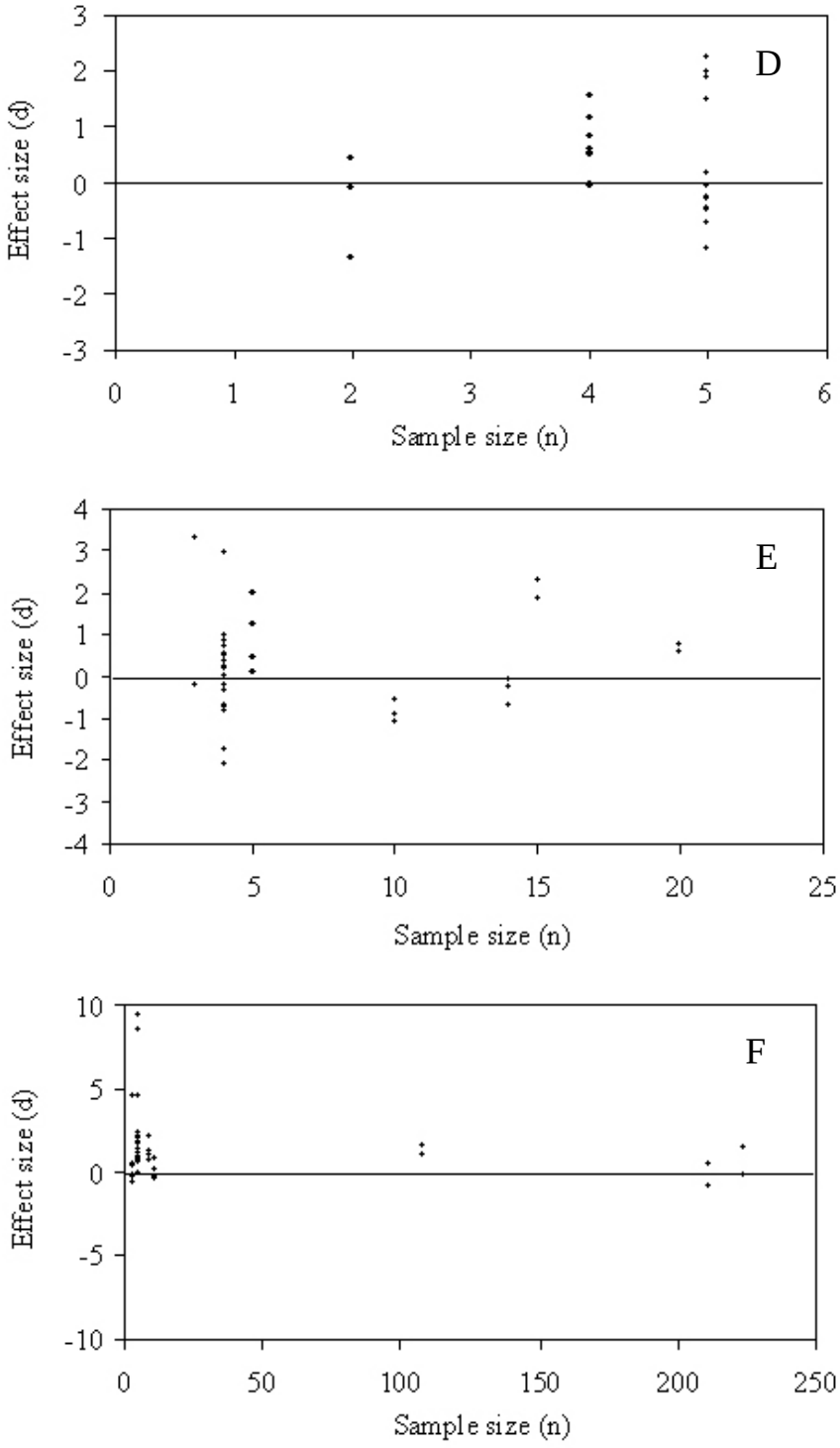
		Weight					Time to hatching				
			95% CI		BiasCI			95% CI		BiasCI	
Group	**Categories**	**E**	**Inferior**	**Superior**	**Inferior**	**Superior**	**E**	**Inferior**	**Superior**	**Inferior**	**Superior**
Family	Alytidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Ambystomatidae	ND	ND	ND	ND	ND	0.8536	0.4341	1.2732	0.4153	1.3067
	Bufonidae	-1.2728	-3.4394	0.8938	-2.3106	-0.0193	ND	ND	ND	ND	ND
	Hylidae	-0.6392	-0.991	-0.2874	-0.9988	-0.3681	ND	ND	ND	ND	ND
	Lymnodynastidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Microhylidae	-0.6129	-1.5231	0.2974	-1.0753	-0.2246	ND	ND	ND	ND	ND
	Myobatrachidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Pelobatidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Pipidae	-0.7476	-1.1382	-0.3569	-1.2448	-0.3258	ND	ND	ND	ND	ND
	Ranidae	-0.286	-0.5066	-0.0653	-0.4673	-0.1101	-0.4521	-1.0092	0.105	-0.768	-0.2049
	Salamandridae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Scaphiopodidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Developmental stage	Embryos	-1.533	-1.8511	-1.215	-1.8883	-1.2388	ND	ND	ND	ND	ND
	Larvae	-0.1852	-0.3461	-0.0244	-0.3217	-0.0289	ND	ND	ND	ND	ND
	Metamorphic	0.1999	-6.044	6.4438	-0.0193	0.4262	ND	ND	ND	ND	ND
	Adults	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Experimental venue	Laboratory	-0.6342	-0.8083	-0.4602	-0.8091	-0.4723	ND	ND	ND	ND	ND
	Mesocosms	0.0132	-0.427	0.4534	-0.3758	0.4361	ND	ND	ND	ND	ND
	Enclosures	0.5734	-0.3378	1.4847	-0.1739	1.228	ND	ND	ND	ND	ND
	Field	0.1588	-1.9113	2.2288	0.0039	0.4262	ND	ND	ND	ND	ND
Pollutant	Nitrogenous compounds	-0.71	-0.926	-0.4939	-0.9099	-0.4846	0.2631	-0.3234	0.8496	-0.2849	0.9464
	Phosphorous compounds	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Pesticides	-0.1992	-0.462	0.0637	-0.3972	0.0341	0.5275	-0.1563	1.2112	0.1242	0.8466
	Heavy metals	-0.5056	-1.2287	0.2175	-1.2123	-0.0056	ND	ND	ND	ND	ND
	Road de-icers	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Wastewater contaminants	0.3985	-0.8743	1.6713	-0.1327	1.1744	ND	ND	ND	ND	ND

APPENDIX 3 (continued). Effect of pollutants on amphibians for each category considered in the *a priori* defined groups for the present study. ND: no data.

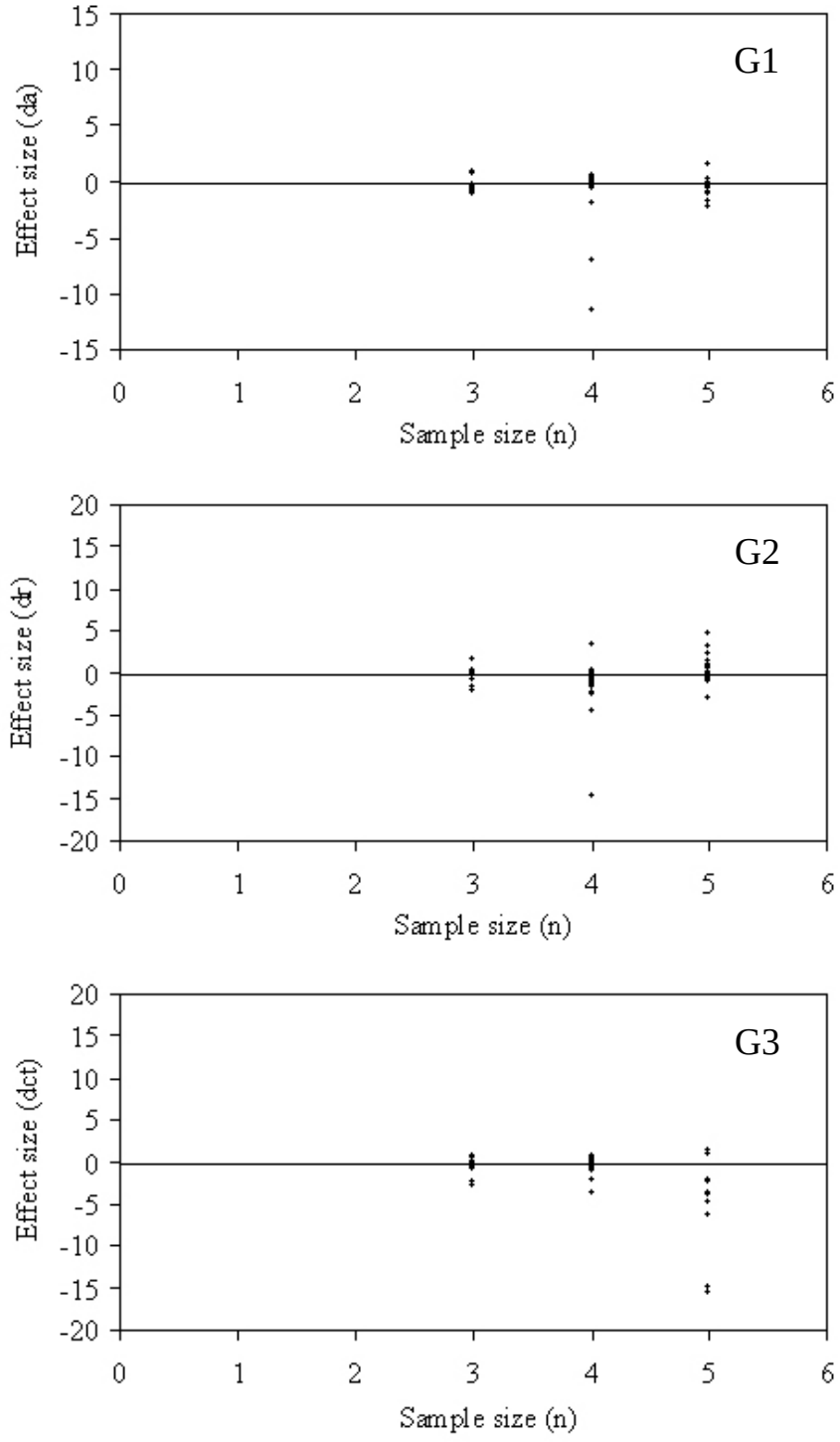
		Time to metamorphosis					Abnormalities				
			95% CI		BiasCI			95% CI		BiasCI	
Group	**Categories**	**E**	**Inferior**	**Superior**	**Inferior**	**Superior**	**E**	**Inferior**	**Superior**	**Inferior**	**Superior**
Family	Alytidae	ND	ND	ND	ND	ND	1.2363	-2.1902	4.6628	0.4204	4.568
	Ambystomatidae	ND	ND	ND	ND	ND	1.4197	0.4768	2.3627	0.7986	2.2141
	Bufonidae	0.6591	-5.5127	6.8309	0.5654	0.7535	1.2388	-0.4754	2.9529	0.8377	1.8911
	Hylidae	-0.0539	-0.8414	0.7336	-0.4088	0.3629	-0.0527	-3.1588	3.0535	-0.2792	0.4024
	Lymnodynastidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Microhylidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Myobatrachidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Pelobatidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Pipidae	ND	ND	ND	ND	ND	0.2174	-2.3588	2.7936	-0.3292	0.7752
	Ranidae	0.354	-0.0472	0.7551	-0.0956	0.8088	1.0827	0.4501	1.7153	0.4918	1.735
	Salamandridae	-0.8678	-2.1456	0.4101	-1.0556	-0.6409	-0.66	-3.7933	2.4733	-0.66	-0.66
	Scaphiopodidae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Developmental stage	Embryos	0.7021	-0.0213	1.4255	0.3975	1.1655	0.9828	0.587	1.3786	0.6293	1.3663
	Larvae	-0.0353	-0.3853	0.3146	-0.3529	0.3327	0.2174	-2.3676	2.8025	-0.3292	0.7752
	Metamorphic	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Adults	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Experimental venue	Laboratory	0.032	-0.4005	0.4645	-0.4013	0.5292	1.0062	0.5773	1.4351	0.6788	1.4375
	Mesocosms	0.3225	-0.2128	0.8578	-0.0085	0.665	ND	ND	ND	ND	ND
	Enclosures	ND	ND	ND	ND	ND	0.5805	-0.4163	1.5772	-0.1534	1.2252
	Field	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pollutant	Nitrogenous compounds	-0.103	-0.8335	0.6276	-0.711	0.9642	0.6954	0.161	1.2298	0.3583	1.068
	Phosphorous compounds	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Pesticides	0.2843	-0.1831	0.7517	-0.0032	0.6445	0.5955	-0.5328	1.7239	0.1149	1.0465
	Heavy metals	0.2055	-0.3831	0.794	-0.1637	0.5962	0.5803	-0.3919	1.5526	-0.1534	1.1685
	Road de-icers	-1.4601	-3.8746	0.9544	-2.1324	-0.6938	ND	ND	ND	ND	ND
	Wastewater contaminants	ND	ND	ND	ND	ND	3.3504	1.8353	4.8655	2.172	5.8678



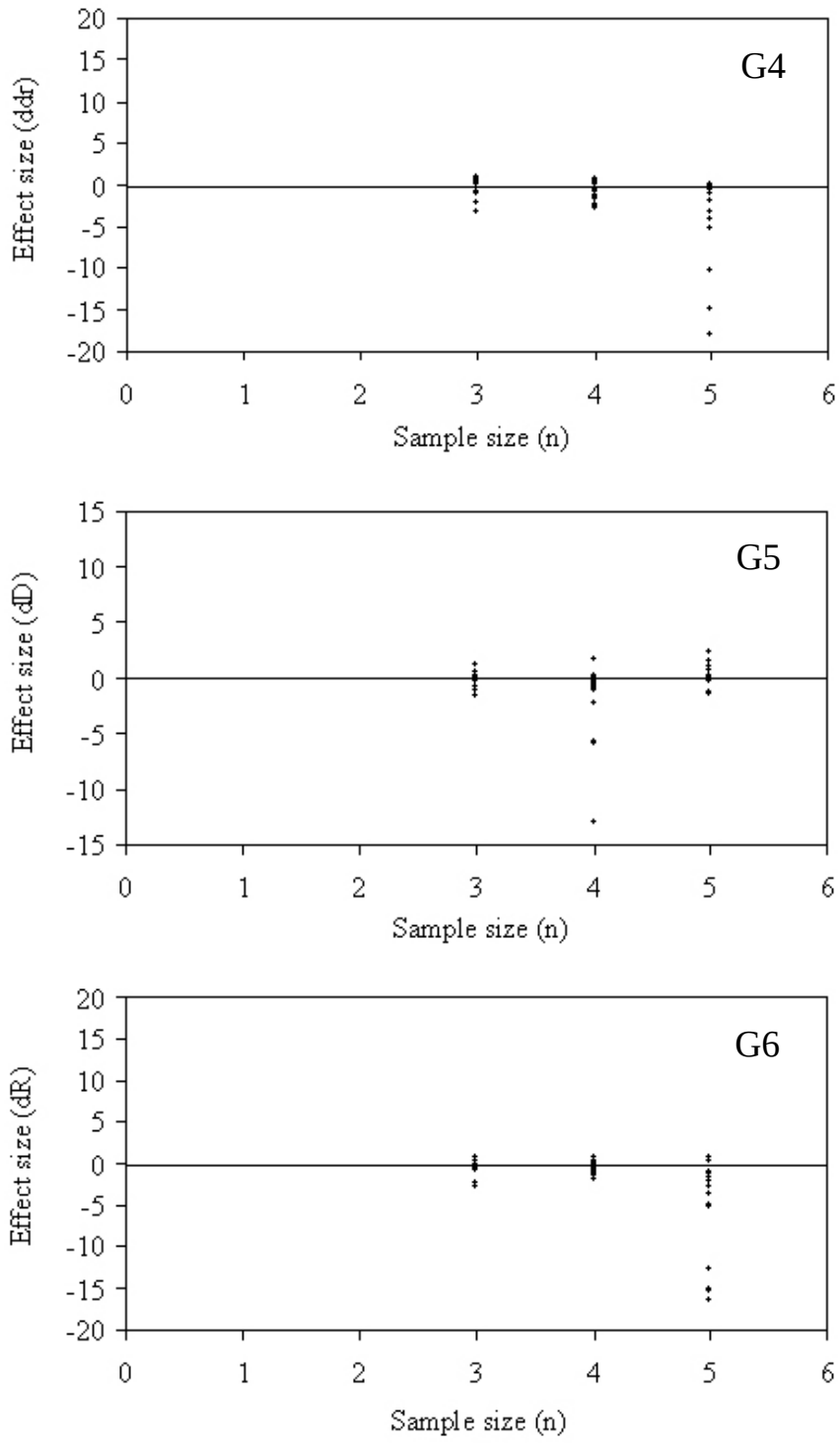
APPENDIX 4. Funnel graphs showing the distribution of effect size (d) as a function of sample size (n). A) Survival (simple meta-analisis); B) Total length; C) Weight; D) Time to hatching; E) Time to metamorphosis; F) Abnormalities; G) Survival (factorial meta-analysis): G1) dp, ns; G2) dp, s; G3) dnp, s, G4) ds, p; G5) dp; G6) ds; G7) di.



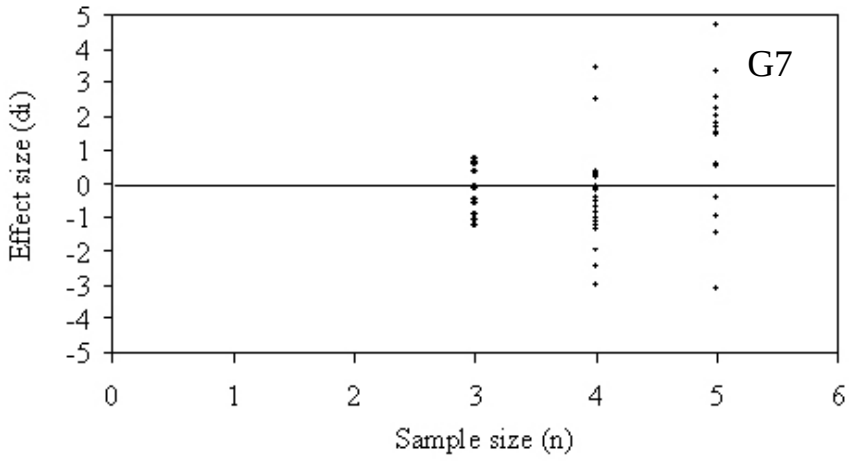
APPENDIX 4 (continued). Funnel graphs showing the distribution of effect size (d) as a function of sample size (n). A) Survival (simple meta-analisis); B) Total length; C) Weight; D) Time to hatching; E) Time to metamorphosis; F) Abnormalities; G) Survival (factorial meta-analysis): G1) dp, ns; G2) dp, s; G3) dnp, s, G4) ds, p; G5) dp; G6) ds; G7) di.



APPENDIX 4 (continued). Funnel graphs showing the distribution of effect size (d) as a function of sample size (n). A) Survival (simple meta-analisis); B) Total length; C) Weight; D) Time to hatching; E) Time to metamorphosis; F) Abnormalities; G) Survival (factorial meta-analysis): G1) dp, ns; G2) dp, s; G3) dnp, s, G4) ds, p; G5) dp; G6) ds; G7) di.



APPENDIX 4 (continued). Funnel graphs showing the distribution of effect size (d) as a function of sample size (n). A) Survival (simple meta-analisis); B) Total length; C) Weight; D) Time to hatching; E) Time to metamorphosis; F) Abnormalities; G) Survival (factorial meta-analysis): G1) dp, ns; G2) dp, s; G3) dnp, s, G4) ds, p; G5) dp; G6) ds; G7) di.



APPENDIX 4 (continued). Funnel graphs showing the distribution of effect size (d) as a function of sample size (n). A) Survival (simple meta-analisis); B) Total length; C) Weight; D) Time to hatching; E) Time to metamorphosis; F) Abnormalities; G) Survival (factorial meta-analysis): G1) dp, ns; G2) dp, s; G3) dnp, s, G4) ds, p; G5) dp; G6) ds; G7) di.

BLOQUE V

CONCLUSIONES GENERALES

CAPÍTULO 10

CONCLUSIONS

(ENGLISH VERSION)

The current Doctoral Thesis provides empirical results obtained after analysing experimentally the impact of nitrogenous compounds mainly on larval stages of the Iberian Water Frog *Pelophylax perezi*, according to an increasing gradient of realism. Moreover, the global impact of chemical pollution on amphibians as a group has been explored by bibliographic synthesis by meta-analysis. Considering the results obtained, the major conclusions reached are as follow, which are organized according to the blocks in which the different chapters were grouped (Block I is not included in the following list of conclusions becaused it deals with the introductory chapters and, consequently, does not show any result).

Block II. Analysis of the effects of nitrogenous compounds on Pelophylax perezi *in laboratory experiments.*

1) The exposure to increasing concentrations of ammonium, nitrite or nitrate, as well as to raising exposure times, significantly increased larval mortality. Consequently, the mean lethal concentration values (LC_{50}) obtained for the nitrogenous ions mentioned decreased as time progressed.
2) LC_{50} values obtained after the exposure to ammonium, nitrite and nitrate for 24 h, 48 h, 72 h and, in the case of ammonium, 96 h showed that mortality was

mainly due to the chronic effect of continuous exposure, rather than to initial exposure.

3) *P. perezi* is rather tolerant to nitrite and nitrate, since the LC_{50} values obtained for these ions are much higher than ecologically relevant concentrations. However, ammonium may threaten this species, since LC_{50} values obtained for this ion are lower than ammonium concentrations detected in the field in the study area.
4) The results obtained suggest that the impact of several nitrogenous compounds should be determined to assess whether a given species is, in general, more tolerant than others. Thus, the tolerance of *P. perezi* tadpoles to nitrate is intermediate as regards others anurans, but the resistance to ammonium and nitrite is generally lower and higher, respectively.
5) The exposure to high concentrations of ammonium, nitrite and nitrate, either isolated or combined, both increased larval mortality and reduced food consumption in relation to the control treatment. Furthermore, when the effects of the nitrogenous ions were analysed separately for each compound, a significant reduction in larval mass was observed at higher ammonium and nitrite concentrations.
6) The exposure to low concentration of ammonium or nitrite reduced the number of censuses that *P. perezi* larvae were detected either at the bottom of the experimental beakers or inactive, respectively. This response was also detected in the case of low concentration of nitrate and of the combination of low nitrite concentration with nitrate and ammonium.
7) The combination of ammonium, nitrite and nitrate produced a more severe impact, compared with the effect of these ions isolated, on larval survival and

food consumption, inactivity level, habitat use and even final mass (in the case of separate analyses). The nature of the effect of nitrogenous mixtures was different according to the response variable studied. Therefore, evidence for a synergistic effect was detected for larval survival and final mass and food consumption, whereas an additive impact was recorded in the case of the behavioral endpoints.

8) Populations of *P. perezi* inhabiting highly nitrogenous polluted habitats were more tolerant than those from less polluted environments. Thus, larvae from highly nitrogenous polluted habitats showed lower larval mortality when they were exposed to high concentration of ammonium, either isolated or combined with nitrite and nitrate. This fact would suggest the potential of *P. perezi* populations to adapt to environments polluted by nitrogenous compounds. However, no overall environment-specific response to nitrogenous compounds was detected in relation to behavior. This disagreement may be a consequence of the different stress that behavioral traits and other lethal and sublethal effects may suffer and which may have masked such environment-specific tolerance to nitrogenous compounds.

9) The presence of ammonium in the water column, either isolated or combined with nitrite and nitrate, did not affect habitat use by juveniles of *P. perezi*, although significant inter-individual variation in treatment avoidance was detected.

Block III. Analysis of the effects of nitrogenous compounds on Pelophylax perezi *in mesocosm and field experiments.*

10) Larval survival was unaffected by ammonium, either isolated or combined with nitrite and nitrate, in mesocosm conditions. However, the exposure to polluted localities significantly increased larval mortality.
11) The exposure to the combination of ammonium, nitrite and nitrate significantly reduced body and tail depth, as well as final mass and growth. Paradoxically, surviving larvae exposed to polluted localities showed higher values for morphological traits, final mass and growth.
12) Neither distance swum by larvae nor swimming speed was affected by nitrogenous compounds or polluted localities.
13) The influence of morphological traits on swimming performance varied greatly across populations and treatments in the case of the mesocosm experiment.
14) The exposure to high ammonium concentration acting alone and to polluted habitats produced a positive trade-off between larval growth and speed, suggesting that the abilitiy to escape from predators depends on how to get larval size (i.e. growth), which is environmentally dependent.
15) Considering the adverse effects produced by pollution on larval survival in the field experiment, it is rather difficult to infer the positive effects that pollution-induced morphologies may represent, as the adaptive hypothesis would suggest.
16) The impact of nitrogenous compounds in the mesocosm experiment and polluted localities in the field experiment may be mediated by the food web, since, in the first case, higher periphyton biomass was detected for the polluted treatments, a

scenario that is also probable in the case of polluted localities, considering the higher nutrient concentration they showed during the study period.

17) Population-specific reponses to nitrogenous compounds were recorded in relation to morphology and larval final mass, suggesting the existence of inter-populational variation in tolerance to nitrogenous pollution and even local adaptation. Nevertheless, populations did not differ in their response in the field localities, which may be due to the fact that field localities were not stressful enough to highlight the eventual divergent responses among larvae from different populations of origin.

18) Larvae of *P. perezi* from different populations of origin possibly had similar vulnerability to predators, since their swimming performance did not differ across populations.

Block IV. Analysis of the effects of pollution on amphibians.

19) Overall, chemical pollution showed a moderate-to-large negative impact on amphibians.

20) Pollution increased larval mortality and the incidence of abnormalities and reduced final amphibian size, although neither time to hatching nor to metamorphosis were affected.

21) The impact of pollution greatly varied between experimental venues, developmental stages and type of pollutant as regards survival, size and abnormality rates.

22) Significant differences among amphibian families and phylogenetic autocorrelation were found only for time to hatching, suggesting that pollutants affect the rate of cellular division in the case of ambystomatids.

23) The interaction among different types of stressors did not significantly affect survival, the exposure to a wide range of biotic and abiotic stressors being more harmful than the exposure to full pollutant stressors.

General conclusions.

24) Chemical pollution is a major threat to amphibians, since both larval survival and other sublethal endpoints are affected.

25) Nitrogenous pollution, in particular, may be an important factor directing the evolution in amphibians.

26) The results obtained in different experimental venues are very heterogeneous, suggesting the great relevance of the context when assessing the impact of a stressor on amphibians.

CAPÍTULO 10

CONCLUSIONES

(VERSIÓN ESPAÑOLA)

La presente Tesis Doctoral muestra los resultados empíricos obtenidos tras analizar experimentalmente el impacto de compuestos nitrogenados principalmente sobre estadios larvarios de la rana común *Pelophylax perezi*, según un gradiente creciente de realismo. Además, se exploró el impacto global de la contaminación química sobre los anfibios como grupo mediante síntesis bibliográfica a través de meta-análisis. Considerando los resultados obtenidos, se exponen a continuación las principales conclusiones extraídas de los mismos, las cuales están organizadas de acuerdo con los bloques en los que los diferentes capítulos fueron agrupados (el Bloque I no está incluido en la siguiente lista de conclusiones debido a que está relacionado con los capítulos introductorios y, consecuentemente, no presenta ningún resultado).

Bloque II. Análisis de los efectos de compuestos nitrogenados en Pelophylax perezi *en experimentos de laboratorio.*

1) La mortalidad larvaria aumentó significativamente con el incremento de las concentraciones y del tiempo de exposición a amonio, nitrito o nitrato. Consecuentemente, los valores correspondientes a la concentración letal media (LC_{50}) obtenidos para los iones nitrogenados mencionados disminuyeron con el tiempo.

2) Los valores LC_{50} obtenidos tras la exposición a amonio, nitrito y nitrato durante 24 h, 48 h, 72 h y, en el caso del amonio, 96 h evidenciaron que la mortalidad se debió fundamentalmente al efecto crónico de la exposición continuada, en lugar de a la exposición inicial.
3) *P. perezi* es bastante tolerante a nitrito y nitrato, ya que los valores LC_{50} obtenidos para estos iones son mucho mayores que concentraciones ecológicamente relevantes. Sin embargo, el amonio puede amenazar a esta especie, ya que los valores LC_{50} obtenidos para este ión son inferiores a las concentraciones de amonio detectadas en el campo en el área de estudio.
4) Los resultados obtenidos sugieren que se debe determinar el impacto de varios compuestos nitrogenados para establecer si una especie dada es, en general, más tolerante que otras. Así, la tolerancia de los renacuajos de *P. perezi* al nitrato es intermedia en relación a otros anuros, mientras que la resistencia al amonio y al nitrito es generalmente mayor y menor, respectivamente.
5) La exposición a altas concentraciones de amonio, nitrito y nitrato, aislados o combinados, incrementó la mortalidad larvaria y redujo el consumo de alimento en relación al tratamiento control. Además, cuando los efectos de los iones nitrogenados se analizaron separadamente para cada compuesto, se observó una reducción significativa de la masa larvaria para altas concentraciones de amonio y nitrito.
6) La exposición a baja concentración de amonio o nitrito redujo el número de veces que las larvas de *P. perezi* se detectaron en el fondo de los contenedores experimentales o inactivas, respectivamente. Esta respuesta se detectó asimismo en el caso de la presencia de baja concentración de nitrato y de la combinación de baja concentración de nitrito con nitrato y amonio.

7) La combinación de amonio, nitrito y nitrato produjo un impacto más severo, en relación al efecto de estos iones aislados, sobre la supervivencia larvaria y el consumo de alimento, nivel de inactividad, uso del hábitat e incluso masa final (en el caso de los análisis realizados separadamente). La naturaleza del efecto de las mezclas nitrogenadas fue diferente en función de las variables de respuesta. Así, se detectaron evidencias de efectos sinérgicos sobre la supervivencia larvaria, masa final y consumo de alimento, mientras que se registró un impacto aditivo en el caso de las variables etológicas.

8) Las poblaciones de *P. perezi* que habitan ambientes altamente contaminados por nitrógeno fueron más tolerantes que aquéllas que ocupan ambientes menos contaminados. Así, las larvas procedentes de ambientes altamente contaminados mostraron menor mortalidad larvaria cuando se expusieron a alta concentración de amonio de manera aislada o combinada con nitrito y nitrato. Este hecho sugeriría el potencial de las poblaciones de *P. perezi* para adaptarse a ambientes contaminados por compuestos nitrogenados. Sin embargo, no se detectó una respuesta general específica del ambiente en relación al comportamiento. Esta discrepancia puede ser consecuencia del diferente estrés que los rasgos etológicos y otros efectos letales y subletales pueden sufrir y el cual puede haber enmascarado esta tolerancia a los compuestos nitrogenados específica del ambiente.

9) La presencia de amonio en la columna de agua, tanto aislado como combinado con nitrito y nitrato, no afectó al uso del hábitat por parte de juveniles de *P. perezi*, aunque se detectó una significativa variación entre individuos en la evitación de los iones nitrogenados presentes en el medio acuático.

Bloque III. Análisis de los efectos de los compuestos nitrogenados en Pelophylax perezi *en experimentos de mesocosmos y campo.*

10) La exposición a amonio no afectó a la supervivencia larvaria, ni aislado ni combinado con nitrito y nitrato, en condiciones de mesocosmos. Sin embargo, la exposición a localidades contaminadas incrementó significativamente la mortalidad larvaria.
11) La exposición a la combinación de amonio, nitrito y nitrato redujo significativamente la altura del cuerpo y de la cola, así como la masa final y el crecimiento. Paradójicamente, las larvas supervivientes expuestas a localidades contaminadas mostraron mayores valores para los rasgos morfológicos estudiados, masa final y crecimiento.
12) Ni los compuestos nitrogenados ni las localidades contaminadas afectaron a la distancia nadada por las larvas ni a la velocidad de natación.
13) La influencia de los rasgos morfológicos en la capacidad natatoria varió en gran medida en función de las poblaciones y tratamientos considerados en el caso del experimento de mesocosmos.
14) La exposición a alta concentración de amonio aislado y a hábitats contaminados produjo una relación positiva entre el crecimiento larvario y la velocidad de natación. Ello sugiere que la habilidad para escapar de los depredadores depende de cómo se alcanza el tamaño larvario (i.e. crecimiento), lo cual es dependiente del medio ambiente.
15) Los cambios morfológicos inducidos por las localidades contaminadas deberían ser beneficiosos para las larvas que los presentan según sugiere la hipótesis adaptiva. Sin embargo, es difícil inferir los beneficios que dichos cambios

representan, ya que la contaminación incrementó la mortalidad larvaria en el caso del experimento de campo.

16) El impacto de los compuestos nitrogenados en el experimento de mesocosmos y de las localidades contaminadas en el experimento de campo pudo estar mediado por la cadena trófica ya que en el primer caso se detectó mayor biomasa de perifiton para los tratamientos contaminados, escenario probable también en el caso de las localidades contaminadas, dada la mayor concentración de nutrientes que presentaron durante el periodo de estudio.

17) Se registraron respuestas específicas de la población de origen a los compuestos nitrogenados en relación a la morfología y masa larvaria final, lo que sugiere la existencia de variación interpoblacional en la tolerancia a la contaminación nitrogenada, e incluso de adaptación local. Sin embargo, las poblaciones no difirieron en su respuesta a las localidades del campo, lo que puede ser debido a que dichas localidades no fueron lo suficientemente estresantes como para inducir las eventuales respuestas divergentes entre larvas procedentes de diferentes poblaciones de origen.

18) Las larvas de *P. perezi* procedentes de diferentes poblaciones de origen posiblemente tuvieron una similar vulnerabilidad a los depredadores, ya que la capacidad natatoria no difirió entre poblaciones.

Bloque IV. Análisis de los efectos de la contaminación en los anfibios.

19) En general, la contaminación química mostró un impacto negativo moderado-grande en los anfibios.

20) La contaminación incrementó la mortalidad larvaria y la incidencia de malformaciones y redujo el tamaño final, aunque no afectó ni al tiempo hasta la eclosión ni hasta la metamorfosis.
21) El impacto de la contaminación varió en gran medida entre condiciones experimentales, estadios de desarrollo y tipo de contaminante para las variables supervivencia, tamaño y tasas de malformación.
22) Se encontraron diferencias significativas entre familias de anfibios y autocorrelación filogenética sólo para tiempo hasta la eclosión, lo que sugiere que los contaminantes afectan a la tasa de división celular en el caso de los ambistomátidos.
23) La interacción entre diferentes tipos de agentes estresantes no afectó significativamente a la supervivencia, siendo la exposición a una amplia gama de agentes estresantes bióticos y abióticos más estresante que la exposición exclusivamente a contaminantes.

Conclusiones generales.

24) La contaminación química es una de las principales amenazas para los anfibios, al afectar tanto a la supervivencia larvaria como a otros parámetros subletales.
25) La contaminación nitrogenada en particular puede ser un importante factor que conduzca la evolución de los anfibios.
26) Los resultados obtenidos en ensayos realizados en condiciones experimentales diferentes son muy heterogéneos, lo que evidencia la gran relevancia del contexto al establecer el impacto de un agente estresante sobre los anfibios.