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*Front cover: Young gopher tortoise (*Gopherus polyphemus*) explores its world on the Archbold Biological Station, Florida, USA. See article on page 25. Photographed by Pablo R. Delis.*

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Predicting *Ambystoma ordinarium* distribution under different climate scenarios in central Mexico

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Global climate change represents one of the most important threats to wildlife populations. Amphibians, specifically salamanders, are particularly susceptible to the effects of a changing climate due to their restrictive physiological requirements and low vagility; however, little is known about which amphibian species are more vulnerable to climate change. Therefore, we aimed to forecast changes in the distribution of the mountain stream salamander, *Ambystoma ordinarium*, using different climate scenarios. Approximately 70 representative presence records were selected to model the current potential distribution and two scenarios based on 2070 climate projections (RCP 2.6 and RCP 8.5) using the MaxEnt algorithm and three global climate models (BCC-CSM1-1, CCSM4 and HadGEM2-ES). A total of three scenarios were simulated using the 10-percentile training presence as the threshold rule. For all scenarios, the average of the area under the receiver operating characteristic curve for the replicated runs was greater than 0.95 ± 0.005 , representing good performance for the current and projected geographical distributions of *A. ordinarium*. Under the most conservative scenario, of a total area of 45,876 km², an average potential distribution area of 5,627 km² was defined for current conditions, decreasing to 4,406 km² for BCC-CSM1-1 in the optimistic scenario (RCP 2.6) and decreasing to 4,020 km² for CCSM4 in the pessimistic scenario (RCP 8.5). The results are useful for the development of future conservation plans, identifying landscapes with high probability to be further affected by climate change and to target potentially resilient habitats that provide consistent climatic conditions for *A. ordinarium* in the face of environmental changes.

Keywords: *Ambystoma ordinarium*; Global climate models; MaxEnt; Salamanders; Species distribution model.

INTRODUCTION

In recent years, amphibians have undergone dramatic declines, and a number of hypotheses exist to explain these declines (Beebee & Griffiths, 2005). Of the 6,682 amphibian species listed on the IUCN Red List in 2018 (IUCN Red List version 2018-1), approximately 41% are threatened with extinction, and, as a group, they are declining more rapidly than birds or mammals, making them the most threatened group of species known to date (Frost, 2018; Jetz & Pyron, 2018; Pyron, 2018). Since the first global assessment of all amphibians was completed in 2004 (Stuart et al., 2004), their situation has worsened. Even when numerous studies have documented the decline in amphibian populations, the complexity of synergistic factors that contribute to the high levels of amphibian imperilment remains unclear (Kerby et al., 2010; Bucciarelli et al., 2014; Campbell et al., 2016). Most of the suggested causes are related to habitat loss and modification, unsustainable exploitation, diseases, pollution, introduction of non-native species and eutrophication of water bodies (Collins & Storer,

2003; Rachowicz et al., 2006; Hayes et al., 2010; Kilpatrick et al., 2010; Jetz & Pyron, 2018; Nowakowski et al., 2018). Climate change has played a significant role in the decline of many species and has been identified as a major and important indirect threat to amphibian populations in recent decades (Pounds et al., 2006). In addition, general climate change scenarios predict a reduction in water availability (Ochoa-Ochoa et al., 2012; 2013), compromising the persistence of aquatic environments and the ability of amphibians inhabiting them to complete their life cycles (Feder & Lynch, 1982; Vörösmarty et al., 2010; Ochoa-Ochoa et al., 2012).

In Mexico, the prioritisation of areas for conservation of biodiversity has traditionally involved natural protected areas (NPAs). The selection of these priority conservation areas has included criteria such as their scenic, historic, cultural and archaeological value or mere availability, with little or no consideration of the geographical distribution of particular biological groups of conservation interest and the variables determining their distributions (Urbina-Cardona & Flores-Villela, 2010). NPAs have usually been established in regions where no previous rigorous place

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prioritisation has been performed (Sánchez-Cordero et al., 2005) but have been the most common instrument used in Mexico for natural resource management and conservation (Bello-Pineda et al., 2013). However, the effective conservation of biodiversity requires not only basic knowledge of this component but also knowledge of the distribution of local and regional biodiversity and those areas where biodiversity is most concentrated to identify the temporal and spatial patterns that govern the fate of biodiversity. Because spatial conservation prioritisation should seek to anticipate the impacts of climate change on biodiversity and how species distributions will respond to such changes, the use of models to infer the distribution of species/populations (e.g., ecological niche models) is an adequate approach to identify priority areas for conservation, management and restoration (Pearson & Dawson, 2003).

Species distribution models (SDMs) coupled to geographic information systems (GIS) have proven to be a powerful tool for predicting potential species distributions (Ruiz-Luna et al., 2017; Escalera-Vázquez et al., 2018). Although these techniques are not new, most of the recent advances have focused on the development of distribution models for plant species, and studies regarding the ecological niche modelling of species related to aquatic systems are scarce (e.g., Domínguez-Domínguez et al., 2006; Zambrano et al., 2006; Contreras et al., 2009; Ruiz-Luna et al., 2017).

Although several studies on ambystomatid species have been conducted in Mexico (Arredondo et al., 2017; Heredia-Bobadilla et al., 2016; 2017; Lemos-Espinal et al., 2016; 2017), few have included *Ambystoma ordinarium* as a study subject. Particularly for *A. ordinarium*, recent reports indicate that populations have declined dramatically (IUCN, 2015), and it is catalogued as endangered by the IUCN Red List of Endangered Species [category B1ab (iii, iv, v)] and is under special protection (Pr) by Mexican Law (NOM-059-SEMARNAT-2001; DOF, 2010). Despite this, almost all studies have been limited to evaluating the abundance, growth and spatial activity (Alvarado-Díaz et al. 2013), diet (Ruiz-Martínez et al. 2014), and phylogeny (Hime et al., 2016) of this species. More recently, Soto-Rojas et al. (2017) studied the effects of habitat quality on morphological abnormalities in this species. Surprisingly, only Escalera-Vázquez et al. (2018) described an approximation of the distribution of *A. ordinarium* using spatial distribution models. Therefore, to accomplish in situ conservation, management and restoration actions, it becomes urgent to identify and prioritize areas where populations of this salamander still exist.

In the present study, we define spatial priorities for conservation to help buffer the impacts of a changing climate using the distribution of *A. ordinarium* as a model. Additionally, we present a spatial analysis based on a climatic niche model with the maximum entropy algorithm to predict the distribution of *A. ordinarium* under different climate scenarios. The information presented here is relevant to prioritising conservation efforts regarding management plans in an area that has experienced a variety of land use changes over the

past century that have enhanced in the last decade, including forest clearing followed by subsequent habitat fragmentation due to agricultural uses and urbanization.

Study area

The distribution of *A. ordinarium* is restricted to the central part of Mexico, encompassing part of the Trans-Mexican Volcanic Belt (TMVB) and limited areas in the higher parts of the Sierra Madre del Sur (Fig. 1). This region presents significant topographic relief and is characterised by an altitudinal range of 1800–3420 m a.s.l., a temperate climate with summer rains, mean annual temperatures of 14–18 °C, and mean annual precipitation of approximately 800 mm (Carlón-Allende et al., 2009). The TMVB is a mass of volcanic rock and other igneous manifestations from the middle to late Miocene, is volcanically active and is considered to be the highest rock-type province in Mexico with the most variation in relief (Gómez-Tuena et al., 2007). On the other hand, the Sierra Madre del Sur is a Mesozoic-Cenozoic magmatic arc in southern Mexico associated with the subduction of the Cocos plate along the Acapulco trench beneath mainland southern Mexico (Ducea et al., 2004).

METHODS

Field survey and Data Preparation

We compiled data on the distribution of *A. ordinarium* from multiple available sources, including VertNet (<http://www.vertnet.org/>), the Global Biodiversity Information Facility (<https://www.gbif.org/>) and localities reported in published papers and the grey literature (e.g., theses, reports, etc.). To improve the accuracy of the modelling outputs, we did not include locality reports with fewer than four decimal places in the latitude/longitude coordinate fields. From these historical records, localities throughout the known geographic range of *A. ordinarium* were visited in a survey conducted from 2013 to 2015 during both the dry and rainy seasons. In each location, surveys were performed from 800–1200 h and 1600–2000 h based on a 300-m downstream and 300-m upstream transect using the location record as the midpoint of the transect. In clear-water locations, we used visual detection and hand nets to capture individuals; on the other hand, if turbidity was high, we used seine nets (2 m long, 4 mm mesh size) every 50 m along the transect depending on the habitat and terrain characteristics (e.g., rocks, pebbles, riparian vegetation); collected individuals were released in situ. Because sampling was focused on recording larvae/and/or paedomorphic individuals and the presence (and not the abundance) of individuals of this species, we considered that the time and date of sampling represent the daily variation in temperature needed to maximise the detectability of this salamander species (Hyde & Simons, 2001). Additionally, most of the mountain streams in this region present characteristics such as a low depth and flow rate and clear water, which allow for better detectability of salamanders in the streams (Soto-Rojas et al., 2017). To identify new/unreported localities for this species, we explored streams and rivers included in the basin that

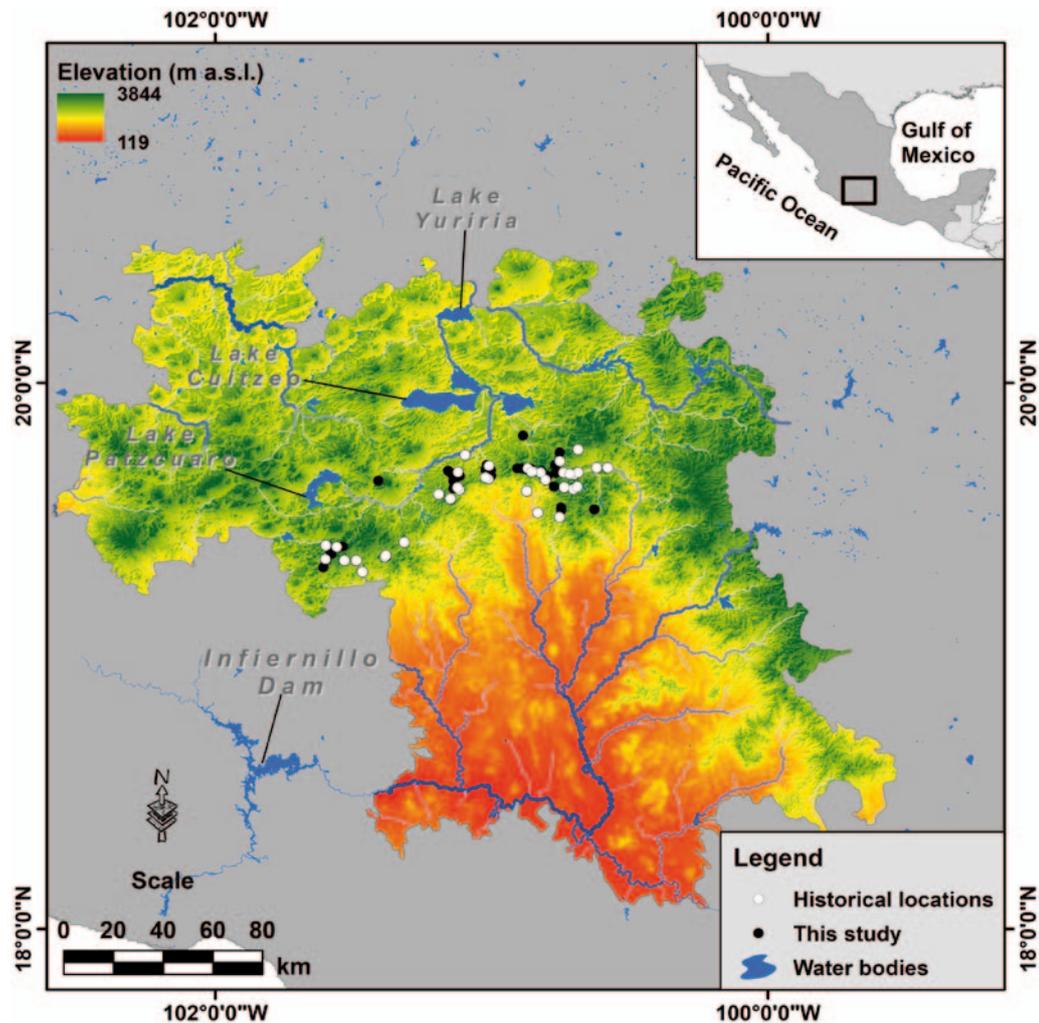


Figure 1. Geographic location of the study area. Dots represent localities for *A. ordinarium* used for modelling its distribution.

were not considered in the historical data. New locations were recorded using a Garmin Montana 680 GPS device (± 2 m accuracy).

Selection of Climate Scenario

We accounted for the known variability in the projected climate data by modelling the climatic niche with three future global climate models (based on projected data to 2070) and uncertainty among mathematical simulations based on the Beijing Climate Center Climate System Model (BCC-CSM1-1, Wu et al., 2013), the Community Climate System Model Version 4 (CCSM4, Gent et al., 2011), and the Met Office Hadley Centre model (HadGEM2-ES, Martin et al., 2011). Climatic models were chosen based on the availability of the projected data in representative concentration pathways (RCPs) 2.6 and 8.5 adopted by the Intergovernmental Panel on Climate Change (IPCC) in its Fifth Assessment Report (AR5). The RCPs represent a range of greenhouse gas concentrations denoted by the amount of radiative forcing (i.e., the difference in the sunlight absorbed by the Earth and the energy radiated back to space) projected into the future compared to pre-industrial values (van Vuuren et al., 2011). RCP 2.6 is considered to be an optimistic scenario in which greenhouse gas concentrations are

projected to decrease over the long term, whereas RCP 8.5 indicates an extreme scenario in which greenhouse gas concentrations are projected to increase drastically in the future (Sutton et al., 2015).

Recent (1950–2000) bioclimatic data and projected climate scenarios (BCC-CSM1-1, CCSM4, HadGEM2-ES) in RCPs 2.6 and 8.5 were available during the implementation of this study. Because we focused on a climatic niche model, we limited our modelling efforts to variables representing temperature and precipitation patterns. These variables were selected because precipitation and temperature show the highest correlation with the movement, migration, and reproductive activity of *Ambystoma* species (Sexton et al., 1990; Palis, 1997; Escalera-Vázquez et al., 2018). For the modelling, we used all 19 bioclimatic variables at 30 arc-second resolution (≈ 1 km) downloaded from the WorldClim global climate data project (version 1.4, release 3; <http://www.worldclim.org/bioclim>; Hijmans et al., 2005). We extracted the values for each environmental variable by location and created a correlation matrix using the raster (Hijmans et al., 2017) and rgdal (Bivand et al., 2018) packages in the R software (version 3.3.1; R Core Team, Vienna, Austria; <https://www.r-project.org/>). To avoid orthogonality of the variables, we removed highly

correlated variables (Pearson method, $r \geq 0.85$) and modelled the distribution of *A. ordinarium* based on the current and projected climatic data. No additional ancillary layers were considered.

Climate Modelling using Maximum Entropy

We constrained our models to the limits of the study area, which are defined by three physiographic subprovinces (Neovolcanica Tarasca, Sierras and Bajíos Michoacanos and Mil Cumbres) of the TMVB together with the subprovince Depresión del Balsas of the Sierra Madre del Sur and delimited by the extreme geographic coordinates: $-102^{\circ} 43.5'$ W and $20^{\circ} 39.5'$ N in the upper left corner and $-99^{\circ} 22'$ and $17^{\circ} 56.5'$ in the lower right. We clipped all BioClim variables using standard geoprocessing techniques within the QGIS 2.16.1 software. This procedure avoids the assumptions of no potential for dispersal or unlimited dispersal (e.g., Milanovich et al., 2010) and reduces potential bias by removing climatic habitats where the species may be unable to disperse.

We used the Maximum Entropy Algorithm (MaxEnt) v3.4.1 program, https://biodiversityinformatics.amnh.org/open_source/maxent/) to model the current and projected distribution of *A. ordinarium*. We chose this model over other correlative presence-only distribution models because of the utility and accuracy of this method as a species distribution modelling technique (e.g., Phillips et al., 2006; Ruiz-Luna et al., 2017; Escalera-Vázquez et al., 2018).

Locality data were randomly divided into training and test sets, which included 75 % and 25 % of the data, respectively. We ran the MaxEnt algorithm under the “auto-features” mode (Phillips & Dudík, 2008) with the default regularisation multiplier value (equal to 1) with a bootstrap replicated runs approach (100 replicates) to obtain a mean estimate of the potential distribution. The maximum number of iterations under which the algorithm converges was set at 1000. We employed the 10-percentile training presence threshold rule, converting continuous models to binary predictions to finally determine the suitable and unsuitable climate distributions (Lemes & Loyola 2013; Escalera-Vázquez et al., 2018). The resulting species distribution-climatic niche was projected onto the current and RCP 2.6 and the RCP 8.5 climate projections. The prediction results of the SDMs were evaluated using the area under the receiver operating characteristic curve (AUC) (Elith et al., 2011) averaged over 100 replicate models.

The impact of climate change was assessed by calculating the change in the suitable area for *A. ordinarium* based on the predicted map of the current climate and each of the future climate change scenarios. To identify the loss/gain areas, we calculated the percent overlap between the current distribution threshold layer and each of the projected thresholded raster cells for each GCM/RCP combination within the QGIS 2.16.1 software.

RESULTS

After data cleaning, 70 locations were included in the modelling processes. Based on the Pearson correlation matrix of environmental variables, the number of layers was reduced from 19 to 8 ($r \geq 0.85$). This provided non-redundant information that was useful for modelling (Table 1). The three main variables were BIO02 - Mean Diurnal Range, BIO06 - Min Temperature of Coldest Month, and BIO14 - Precipitation of Driest Month (Table 1). BIO02 was the environmental variable with the highest contribution in almost all simulations (>30 %).

The mean AUC for the *A. ordinarium* distribution models based on the current climate was 0.966 (range = 0.909–0.993; SD = 0.017), with the AUC being significantly better than random. The mean AUC that showed the lowest value varied depending on the global climate model and the RCP considered, with the mean AUC = 0.969 (range = 0.924–0.990; SD = 0.013) for CCSM4 in the “optimistic” scenario and the mean AUC = 0.967 (range = 0.909–0.990; SD = 0.016) for HadGEM2-ES in the “pessimistic” scenario.

As stated above, our study area is constrained by three physiographic subprovinces of the TMVB and one subprovince of the Sierra Madre del Sur, together accounting for an area of 45,876 km² with elevations between 120 and 3,850 m a.s.l. (mean = 1,685 m and std. dev. = 734 m) and a slope ranging from 0 to 80° (mean = 12° and std. dev. = 10°). Agriculture and evergreen forest are the two most prevalent land cover types, covering approximately 65 % of the study area.

Using the 10-percentile training presence (10 ptp) as the threshold rule, we obtained an average area of 5,630 km² (12.3 % of the total study area) for the potential distribution of this species. Our results suggest the presence of three main fragments with high habitat suitability for *A. ordinarium* and several isolated patches of minor extent (Fig. S1). The greatest patch is in the central mountain part of the study area (approximately 19.7° N, -100.9° W), a second patch is located to the south of Lake Patzcuaro (approximately 19.4° N, -101.5° W), and a third patch follows a narrow band through the central and south-eastern part of the mountain system (approximately 19.1° N, -100.0° W). Approximately 98 % of the predicted area is within an elevation range of 1,900 to 3,000 m a.s.l. (mean = 2,382 m and std. dev. = 262 m).

According to the Land Use and Vegetation Chart (Series VI; scale 1:250,000; edition 2017) published by the National Institute of Statistics and Geography (INEGI according to its name in Spanish), the main land cover and land use within the predicted area is evergreen forest (62.5 %), followed by agriculture (26.7 %). It is important to mention that of the 3,515 km² of forest, 731 km² has already shown some degree of disturbance (Fig. S1).

The high-suitability area calculations for the future varied depending on the GCM and RCP considered. A decrease to 4,410 km² is expected for BCC-CSM1-1-Optimistic Scenario (RCP 2.6). The greatest loss in habitat is predicted using the CCSM4 Global Climate Model with RCP 8.5, decreasing to 4,020 km². The projected mean

change in the *A. ordinarium* suitable climatic habitat size by 2070 varied depending on the GCM and RCP used, even the most “optimistic” scenario projected an approximately 15 % reduction in the suitable climatic range, mostly distributed in the east and south-southeast (Table 1; Fig. 2).

Although a habitat reduction is predicted for all scenarios, the eastern part of Lake Patzcuaro, the south-eastern part of the study area and all isolated patches are predicted to lose the greatest area. For all simulations, the area expected to remain with no change is approximately 2,700 km², while the projected climatic habitat loss by the year 2070 (only predicted in the current scenario) is approximately 1,880 km² (Fig. 3).

DISCUSSION

In this study, we modelled the potential geographical distribution of *A. ordinarium* in central Mexico under current and future climate scenarios and highlighted that future climate change might not only reduce the distribution range of *A. ordinarium* but also increase the fragmentation in the region, producing isolated populations.

Regarding the environmental variables considered in this study, some authors suggest that biotic interactions among species and the ability of species to disperse may lead to erroneous results using only models containing environmental-bioclimatic variables (Woodward & Beerling, 1997; Davis et al., 1998). However, a recent study by Bucklin et al. (2015) indicated that the incorporation of additional predictors has relatively minor effects on the accuracy of climate-based species distribution models. Therefore, although there are more variables available (e.g., solar radiation, elevation, slope), we agree with Duan et al. (2016) that the addition of other climatic variables would increase the danger of model over-fitting. Thus, given the low dispersal ability of *A. ordinarium* and its semi-permeable skin that makes it sensitive to changes in the thermal and hydric environment (Ochoa-Ochoa et al., 2012), we can consider higher correlations with variables that are more functionally related to biological traits, such as temperature and rainfall (e.g., Beneski et

al., 1986; Homan et al., 2008; Contreras et al., 2009). In addition, the environmental variables considered in this study are critical factors in all global climate models.

Although previous studies have suggested the potential importance of elevation in defining salamander distributions (Dillar et al., 2008; Searcy & Shaffer, 2014), our study excluded this layer following the recommendations of Elith & Leathwick (2009), who consider that elevation rarely directly affects the distribution of species. For example, in the studied area, an increase in elevation is generally related to higher annual precipitation and lower annual temperatures. Therefore, high-elevation areas may indirectly provide the temperate/moist environments required for *A. ordinarium* and amphibian species that depend on moisture for cutaneous respiration when metamorphosis occurs. In addition, the reduced set of environmental predictors used were generated at a regional scale, similar to other previous studies in which the distributions of salamanders were modelled (Rissler & Apodaca, 2007; Milanovich et al., 2010; Sutton et al., 2015). In this regard, our models generated predictions over very large areas by using spatial data that were readily available. Therefore, the methodology applied here can be easily adapted to predict the distributions of other paedomorphic ambystomatid species related to streams at a regional scale (e.g., *A. altamirani*, *A. leorae*, *A. rivulare*).

Although a plethora of information exists about *Ambystoma* spp. in Mexico, only a few studies have explored the use of SDMs for predicting their geographical distribution. Contreras et al. (2009) attempted to predict the species distribution of *A. mexicanum* (axolotl) at a local scale, generated microclimatic and water quality layers at high resolution (1 m²) and used them as predictors. They argued that the recorded variables for Lake Xochimilco were not sufficiently fine to represent the actual heterogeneity of the system. In the present study, this approach was not possible due to the wider range of the natural distribution of *A. ordinarium* and equipment limitations. Particularly for *A. ordinarium*, Escalera-Vázquez et al. (2018) made a first approximation of the spatial distribution of this species. According to their results, the area predicted by the model is characterised

Table 1. Bioclimatic variables and its contribution (%) in the modelling of the potential distribution of *A. ordinarium*. Bold numbers represent the variables that have higher percent of contribution. Estimated area is in km² and figures are rounded to the nearest tens. The RCP 2.6 represent the Optimistic Scenario; whereas the RCP 8.5 represents the Pessimistic Scenario. Global Climatic Models are: BC = BCC-CSM1-1; CC = CCSM4; HG = HadGEM2-ES.

| Bioclimatic variable | Current | RCP 2.6 | | | RCP 8.5 | | |
|---|-------------|-------------|-------------|-------------|-------------|-----------|-------------|
| | | BC | CC | HG | BC | CC | HG |
| BIO2 - Mean diurnal range (Mean of monthly (max temp - min temp)) | 35.4 | 32.3 | 38.2 | 32.6 | 31.9 | 30 | 30.6 |
| BIO3 - Isothermality (BIO2/BIO7) (*100) | 3 | 0.2 | 0.8 | 0.5 | 2.2 | 2.7 | 6 |
| BIO4 - Temperature seasonality (standard deviation *100) | 8 | 6.3 | 5.9 | 9.2 | 4.9 | 8.2 | 4.1 |
| BIO6 - Min temperature of coldest month | 21.4 | 13.7 | 28.9 | 21.8 | 20.8 | 22 | 10.5 |
| BIO12 - Annual precipitation | 2.9 | 3 | 2.7 | 2.8 | 3.6 | 3.3 | 2.2 |
| BIO14 - Precipitation of driest month | 21.3 | 34.7 | 12.9 | 20.9 | 30.9 | 23.7 | 35.1 |
| BIO15 - Precipitation seasonality (Coefficient of Variation) | 6.9 | 8.3 | 9.6 | 11.2 | 5 | 9.3 | 8 |
| BIO18 - Precipitation of warmest quarter | 1.2 | 1.5 | 1.1 | 1 | 0.7 | 0.7 | 3.5 |
| Area (km ²) | 5630 | 4410 | 4820 | 4640 | 4550 | 4020 | 5220 |

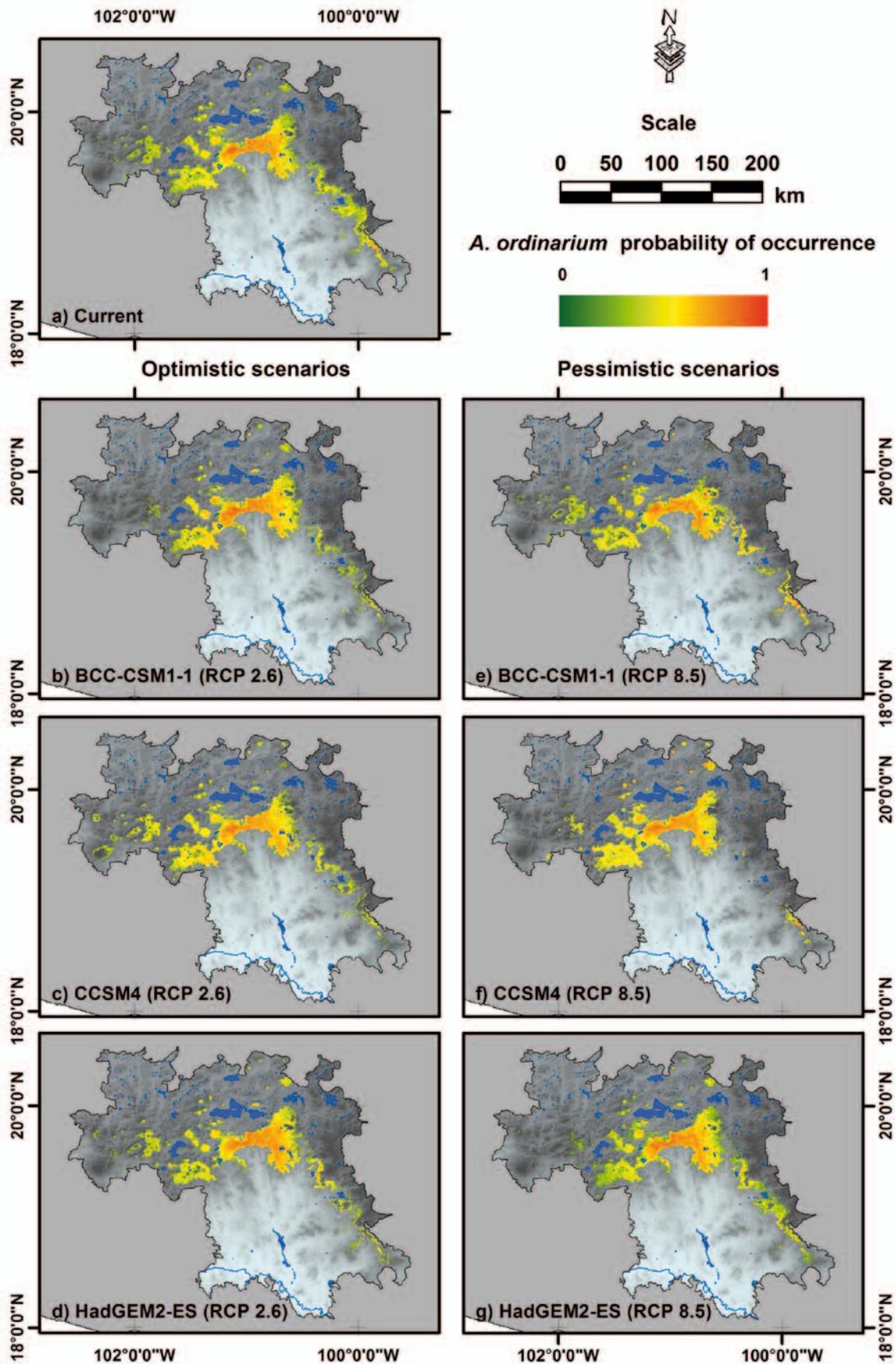


Figure 2. Potential distribution models for the Michoacán stream salamander (*A. ordinarius*) calculated for both current and projected scenarios with three global climate models (BCC-CSM1-1, CCSM4 and HadGEM2-ES) and two representative concentration pathways (RCP 2.6 and 8.5). Warmer colours show areas with higher probabilities of occurrence.

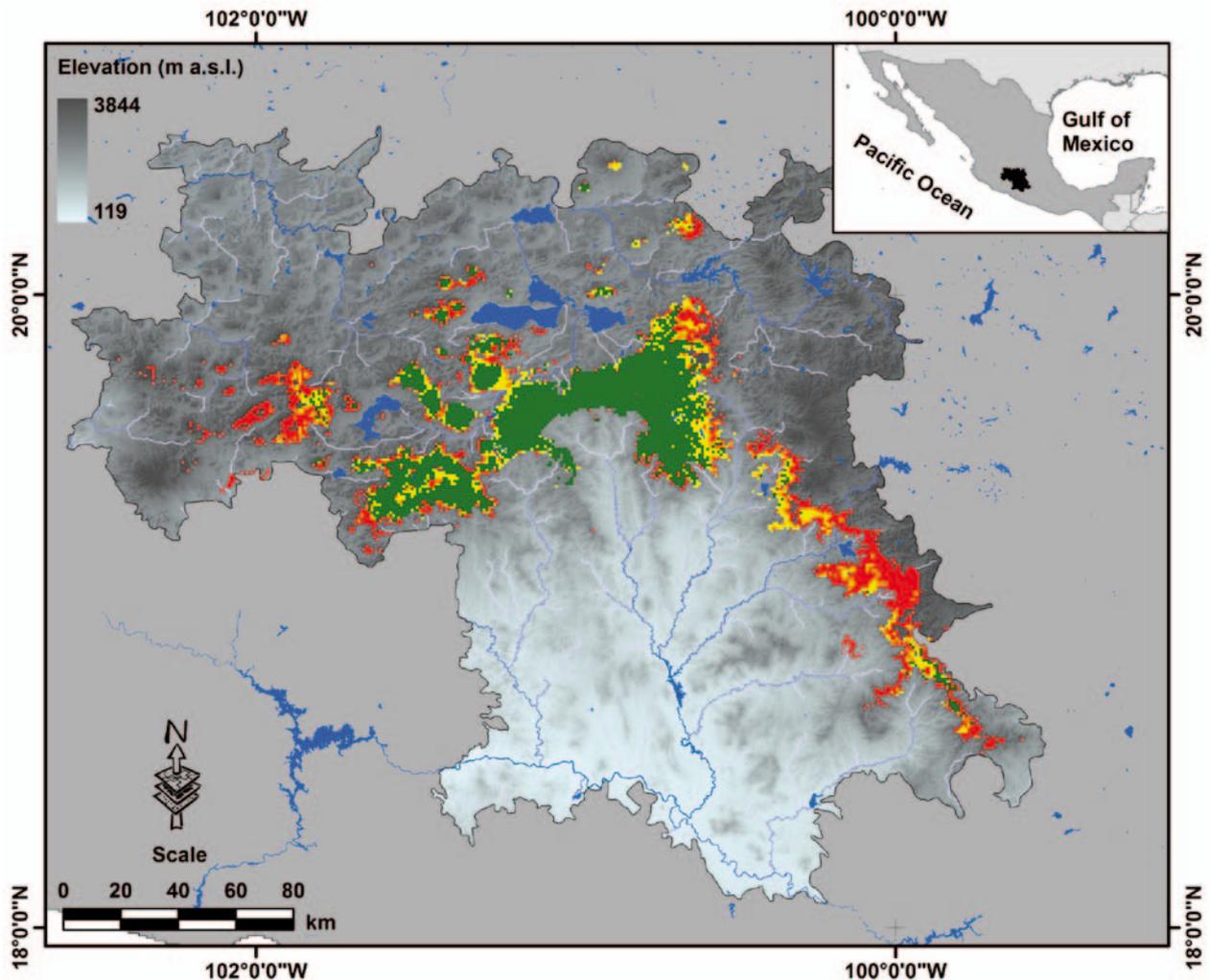


Figure 3. Simulated distribution of suitable climate space for *A. ordinarium* based on the overlap between current and projected distribution models. Green represents areas of no change, yellow represents areas predicted by at least two models, and red represents lost area.

by higher altitudes and irregular topography with forest in the central and south-eastern part of the total distribution area of the species, highlighting a reduction of nearly 253 km² of forest in 16 years (1997-2013). According to our results, if the trend detected by Escalera-Vazquez et al. (2018) continues, an additional reduction of 901 km² in forest is expected by 2070. However, none of these studies forecasted potential future distributions under various climate scenarios to understand the possible responses of amphibians to future climate change.

Although the IUCN recommends that the extent of occurrence is quantified by drawing a minimum convex polygon around known or inferred presence localities, the use of SDMs has already been applied to inform IUCN Red List assessments (de Castro et al., 2014; Syfert et al., 2014). Our findings suggest that the current predicted extent is greater than the extent of occurrence for *A. ordinarium* reported by the IUCN (4,357 km²). This result is consistent with that reported in Syfert et al. (2014) and provides useful additional information about how much larger the actual extent of occurrence could be and where it is likely to extend geographically. In contrast with the methodology recommended by the IUCN, we used data from multiyear resurveys for higher confidence,

making the model more reliable (de Castro et al., 2014). Additionally, as Kerby et al. (2010) suggested that amphibians are more resilient to anthropogenic changes than expected, the assumption of the dramatic decrease in *A. ordinarium* populations could be a misinterpretation of data obtained from low-effort surveys.

Our results provide information on a species highly associated with aquatic ecosystems, suggesting that the major problems that this species will face in the next 50 years are those related to climatic change and water scarcity (Rissler & Apodaca, 2007; Dillars et al., 2008; Contreras et al., 2009; Milanovich et al., 2010). In addition, regional climate change over the long term represents the greatest threat to the persistence of resilient landscapes that amphibians inhabit (Suazo-Ortuño et al., 2015). The TMVB is one of the most ecologically degraded areas in Mexico, which is related to the proximity to highly urbanised areas, and is heavily impacted by agricultural activities (Sunny et al., 2017). Our findings are in accordance with those reported by Sunny (2017) and suggest that the TMVB is vulnerable to effects from climate change, as evidenced by a relatively large projected loss of the *A. ordinarium* climate niche.

It has been suggested that due to their low dispersal

capacities and small home ranges, almost all *Ambystoma* species in the TMVB are threatened by habitat loss, fragmentation, the contamination of rivers and lakes and the introduction of nonnative species (Heredia-Bobadilla et al., 2016; Soto-Rojas et al., 2017; Estrella-Zamora et al., 2018). On one hand, habitat transformation can result in small, isolated and fragmented populations, increasing the chance of inbreeding and lower genetic variability (Heredia-Bobadilla et al., 2016). On the other hand, the introduction of non-native species can help spread emerging amphibian diseases. Indeed, the results found by Estrella-Zamora et al. (2018) suggest that *A. altamirani* is less abundant in the presence of *Oncorhynchus mykiss*, probably associated to higher predation and consumption on salamander larvae by this trout species (McGarvie & Cox, 2007). Particularly for *A. ordinarium*, Soto-Rojas et al. (2017) recorded the presence of *O. mykiss* at three sites in the 16 streams sampled in the TMVB. These studies demonstrated a strong negative association between nonnative *O. mykiss* and amphibians.

Considering that *A. ordinarium* occurs in habitats adjacent to riparian habitats, the potentially high vulnerability of this species to climate change suggests the need for the implementation of conservation measures (e.g., maintenance of riparian forest as a buffer area as well as the conservation of habitat connectivity) to mitigate potential climatic impacts. Indirectly, our results highlight the importance of taking immediate conservation, management and restoration actions in this region, where anthropogenic impact is high, mainly related to the increase in the area used for plantations (e.g. avocado), urbanisation, and deforestation at higher elevations. Likewise, our pessimistic model should be considered because central Mexico is a hotspot for problems associated with water security and biodiversity loss in rivers in the upcoming decades (Vörösmarty et al., 2010). In this study, we used *A. ordinarium* as a model species in indirectly identify environments where high levels of interaction between terrestrial and aquatic ecosystems occurs. This information is useful for the implementation and development of restoration and conservation plans. Although long-term solutions to climate change are complex and seemingly inaccessible to many land managers, strategic conservation planning is a proactive and tangible approach to providing climate refugia (Sutton et al., 2015). Such efforts should be oriented towards tight coordination among local people, authorities, and academia to promote long-term sustainable management.

Finally, although our models were generated with a sufficiently high discriminatory power to be useful and could be considered better than the results obtained by chance (e.g., AUC > 0.7, Ruiz-Luna et al., 2017), our results should be considered with caution because a) many factors other than climate itself play an important role in determining species distributions over time (Pearson & Dawson, 2003), b) the low number of presence data used for training could have an influence on the model performance, and c) no observations of species occurrence are available from the training data

to directly support the projected predictions (Elith & Leathwick, 2009).

CONCLUSIONS

Our study represents the first regional attempt to predict the range of distribution of *A. ordinarium* and its changes under future climate scenarios. Overall, our study provides a conservation tool that can be used to identify priority habitats for *A. ordinarium* conservation. The spatial limits detected here widen the traditional limits reported by the IUCN. Understanding the spatial distributions of threatened, endangered, and sensitive amphibian species and their habitat occupancy is an important component of regional conservation planning. If this information is readily available to managers of large and heterogeneous landscapes, conservation planning efforts will be more informed and potentially more effective.

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Translocation of a large population of great crested newts

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The great crested newt, *Triturus cristatus*, is a European Protected Species. Its legal protection requires appropriate mitigation and compensation measures to be developed for populations threatened by built development in order to demonstrate no likely detriment to the maintenance of the favourable conservation status of the species in its natural range. A major regeneration project at a site near Neath Port Talbot in South Wales, United Kingdom, resulted in the destruction of the breeding sites and associated terrestrial habitats of a population of great crested newts. Capture and translocation of 9,500 newts of all life stages was undertaken between 2009 and 2016 to an adjacent receptor site with waterbodies and extensive terrestrial habitats. Eggs and larvae have been observed in waterbodies in the receptor site throughout the monitoring period from 2013 to 2017, and great crested newts are colonising new waterbodies. The translocated population was much larger than the population size predicted from the baseline surveys of waterbodies on the development site. The design, planning and licensing of mitigation and compensation schemes for great crested newts threatened by built development projects need to give full weight to the limitations of survey methods when estimating the size of a population.

Keywords: amphibian, European Protected Species, great crested newt, translocation, *Triturus cristatus*

INTRODUCTION

The great crested newt (*Triturus cristatus*) is one of six species of crested newt in Europe and its global distribution extends as far as central Asia (Jehle et al., 2011). This species, like other amphibians, requires suitable ponds for breeding in proximity to high quality terrestrial habitats for foraging in summer and hibernation in winter (Langton et al., 2001; Jehle et al., 2011). The great crested newt is threatened and declining in the United Kingdom and western Europe (Langton & Burton, 1997; Jehle et al., 2011; Denoel, 2012).

The great crested newt has full legal protection as a European Protected Species through the EC Habitats Directive (Council Directive 92/43/EEC) which is implemented through national legislation by member states of the European Union. The legal protection afforded to the great crested newt has resulted in the capture and translocation of populations that are threatened by built development that has been granted consent, but where there is no alternative to translocation (such as avoiding the loss of aquatic breeding sites or resting places in terrestrial habitats), in order to ensure that the favourable conservation status of the species in its natural range is maintained.

A recent review of great crested newt mitigation measures in England and Wales concluded that there was insufficient evidence to allow general conclusions

about the effectiveness of such interventions (Lewis et al., 2017). Reports of translocations of great crested newts (Herpetofauna Consultants International, 2007; Redgrave, 2009; Gustafson et al., 2016) focus on determining the success of the translocation through monitoring of the translocated populations rather than reviewing the translocation process. Edgar et al. (2005), Gustafson et al. (2016) and Lewis et al. (2017) emphasise the crucial role of reporting and evaluating translocations of great crested newts in order to develop appropriate and effective methods for mitigation and compensation for the loss of terrestrial and aquatic habitats from built development projects. More generally, Germano et al. (2015) conclude that there is a failure to document the outcomes of translocations of animals, including amphibians, as part of mitigation for human activities in contrast to translocations undertaken for conservation reasons.

A capture and translocation programme for great crested newts was undertaken between 2009 and 2016 for a major regeneration and redevelopment project at Coed Darcy in South Wales on the site of a decommissioned and demolished oil refinery (Box et al., 2010) (Fig.1). Extensive treatment and remediation of contaminated soils was required involving the destruction of the newt breeding sites and associated terrestrial habitats before redevelopment of the site for residential and commercial uses. It was necessary

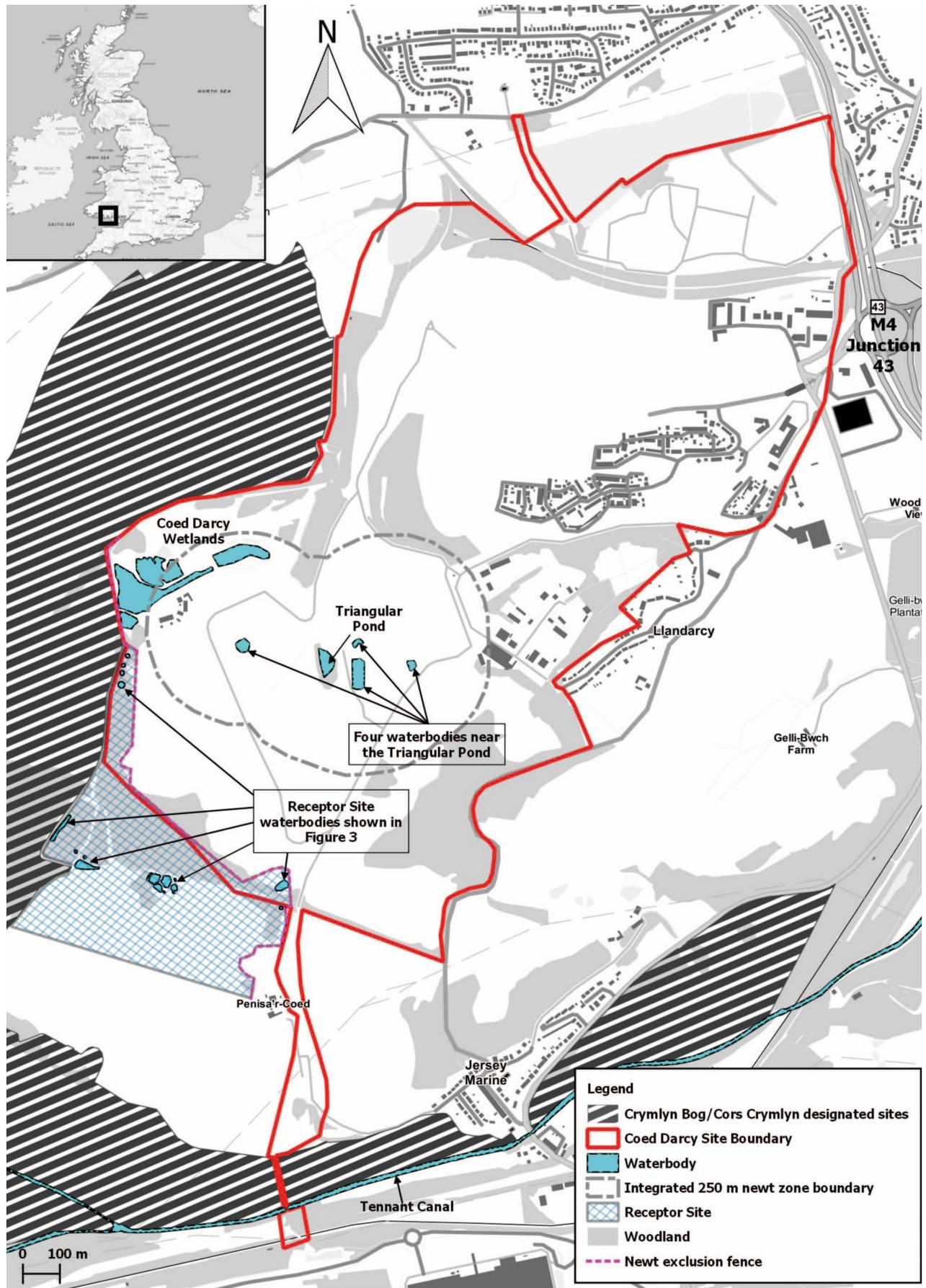


Figure 1. Location of the main Coed Darcy site showing the key waterbodies and the Receptor Site for great crested newts together with the adjacent Cors Crymlyn/Crymlyn Bog

to demonstrate that there was no feasible alternative to the translocation scheme as required by guidance (Joint Nature Conservation Committee, 2003). This paper sets out the lessons learned from one of the largest translocations of great crested newts in Britain with the aim of augmenting the evidence-base for those making decisions about built and infrastructure developments that involve newt habitats and increasing the effectiveness of future mitigation and compensation schemes.

SITE DESCRIPTION AND METHODS

Coed Darcy site

Coed Darcy is an urban development of around 250 ha comprising residential, business and commercial uses, education and other community facilities together with woodlands and landscaped areas being undertaken by St. Modwen Developments Ltd. (St. Modwen) on the site of the former British Petroleum (BP) Llandarcy oil refinery in South Wales (Fig. 1). The site has a complex topography resulting from its industrial past and was dominated by scrub vegetation in association with oak/birch woodland, wet woodland with alder and willow, ephemeral/short perennial vegetation, tall ruderal vegetation, bare ground, spoil and the remnants of oil refinery infrastructure which included large piles of rubble. Coed Darcy is adjacent to the internationally important Crymlyn Bog/Cors Crymlyn Special Area of Conservation, Wetland of International Importance (Ramsar site), National Nature Reserve and Site of Special Scientific Interest.

Great crested newts were recorded at Coed Darcy in only one waterbody (the Triangular Pond) (Fig. 1) as 15 newts in 2001 (torchlight survey on two occasions from April to May) and one newt in 2003 (egg search, torchlight survey and bottle trapping on four occasions from March to June) (Parsons Brinckerhoff, 2005). The 2001 survey was undertaken before publication of the standard survey methodology for great crested newts (English Nature, 2001) and was a much simpler survey. The 2003 survey followed the standard presence/absence methodology in this guidance but was constrained by dense scrub around one waterbody. This population was at the lower end of the 'medium' population size class (defined as 11 to 100 adult newts (English Nature, 2001)) and was considered to be an isolated population that was either a relict or an introduced population (Parsons Brinckerhoff, 2005).

Great crested newts were confirmed in 2008 by Atkins using standard population surveys involving torchlight surveys and bottle trapping on six visits between March and June (English Nature, 2001) in the Triangular Pond as a 'medium' population (the maximum adult count was 15 great crested newts) and as 'small' populations (defined as up to 10 adult newts (English Nature, 2001)) in each of four vegetated waterbodies near the Triangular Pond (Fig. 1). These surveys were constrained because three of the four waterbodies associated with the Triangular Pond were too shallow to use bottle traps and because scrub along the shorelines of some waterbodies was so dense that access to the waterline was not possible

(e.g. only 10 % of the shoreline of the Triangular Pond was accessible). The maximum distance between the five waterbodies was 430 m and the great crested newt metapopulation was assessed as being a 'medium' population size class with a cumulative peak count of 15 adult newts summed across the five waterbodies on the same survey date. Great crested newts were not recorded in a comprehensive presence/absence survey in 2008 of the other waterbodies on the Coed Darcy site and all the waterbodies within 500 m of the site boundary.

The Triangular Pond, the main breeding site, was approximately 900 m² in area at the water level and 1-1.5 m in depth with abundant broad-leaved pondweed (*Potamogeton natans*) and rigid hornwort (*Ceratophyllum demersum*), frequent white water-lily (*Nymphaea alba*) and occasional common water-starwort (*Callitriche stagnalis*) and bulrush (*Typha latifolia*) (Fig. 2). The Triangular Pond is a distinct feature on two aerial photographs dated 1923 (the oil refinery was opened in 1922) and one dated 1947 (Aerofilms Ltd./Britain from Above). It had an artificial bund on its two eastern sides and was probably constructed to impound surface water runoff as a source of fire-fighting water for the refinery fire station which was based at that time in the southern tank farm that contained the crude oil storage tanks; the Triangular Pond continued in this role in the 1960s during



Figure 2. Marginal aquatic vegetation in the Triangular Pond

the substantial expansion of the refinery (John Smith, BP & St. Modwen, pers. comm., 6 February 2017).

There was no alternative to the capture and translocation of the great crested newts (Joint Nature Conservation Committee, 2003) because treatment and remediation of the contaminated soils involved destruction of the Triangular Pond and associated terrestrial habitats. The land around the Triangular Pond had a history of contaminative uses and 'special process areas' were identified for particular attention during remediation in agreement with the regulator Environment Agency Wales, now Cyfoeth Naturiol Cymru/Natural Resources Wales (NRW). The Triangular Pond and surrounding areas required excavation to allow for the treatment of hydrocarbon and heavy metal

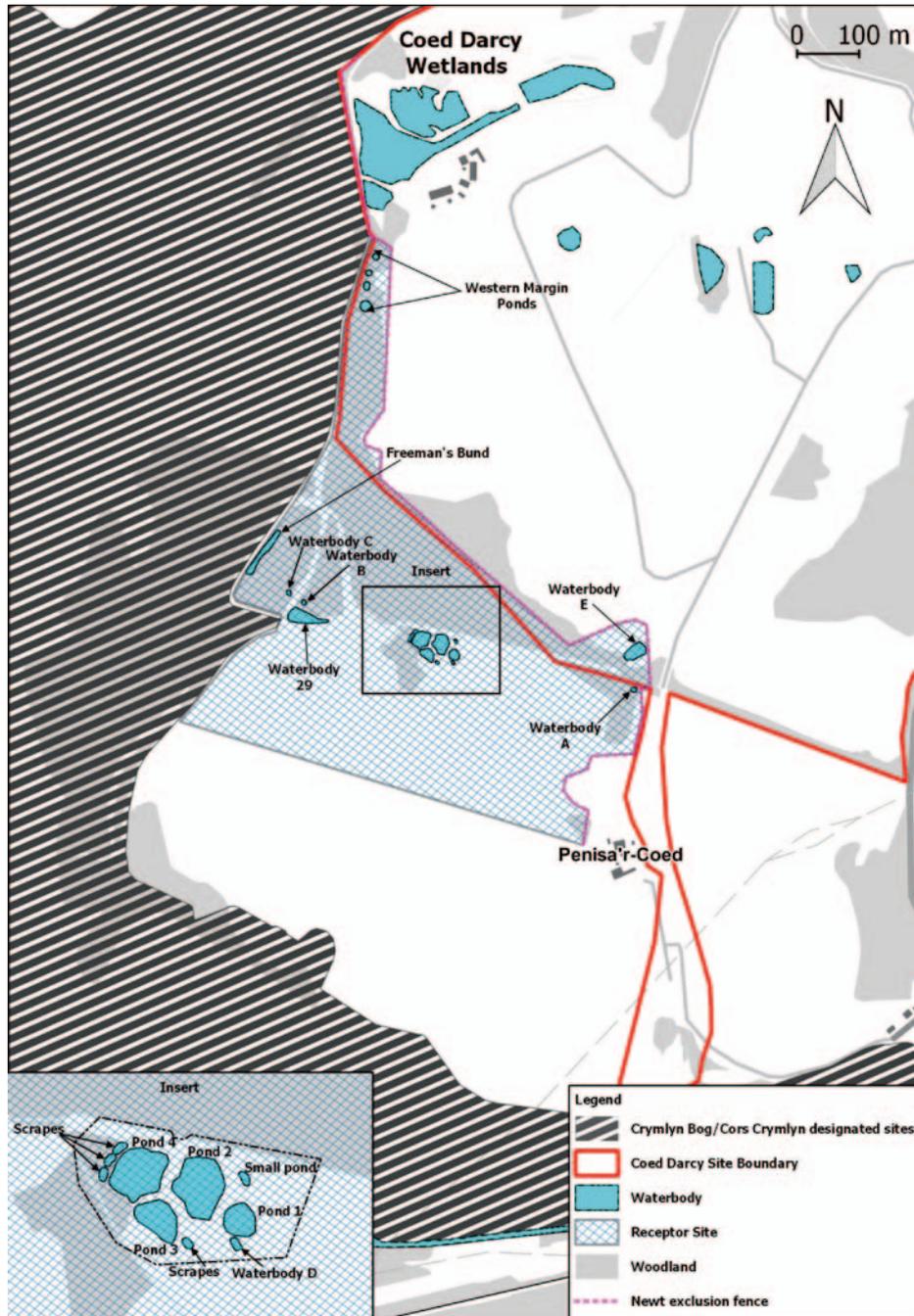


Figure 3. Great crested newt Receptor Site

contamination.

Receptor Site

The Receptor Site (Fig. 3) was established in 2010 adjacent to the Coed Darcy site on terrestrial and aquatic habitats including rush pasture, lowland heath and mire, semi-improved acid grassland, scrub and bracken (*Pteridium aquilinum*), wet woodland and open water. Great crested newts were not present in the existing waterbodies in the Receptor Site prior to the newt translocation. New waterbodies were created in autumn 2010: these were Ponds 1-4, Small pond, Waterbody D and the Scrapes shown in Figure 3; one of the new waterbodies is shown in Figure 4. Extensions to the original Receptor Site were required in 2011, 2015 and 2016 to give a final area of 26.3 ha in conjunction with the creation of further ponds and scrapes in response to the greater than expected numbers



Figure 4. Pond 4 in the Receptor Site in September 2012 following creation in autumn 2010

of great crested newts that had been captured. Habitat management works were undertaken to the existing waterbodies involving vegetation clearance in and around the waterbodies and tree felling to reduce shading. Amphibian refuges/hibernation sites were created using sandstone blocks, rocks, logs, tree stumps and soil.

A sturdy plastic panel newt fence was erected along the boundaries of the Receptor Site with the Coed Darcy site, and along its boundary with the route of the proposed Southern Access Road to Coed Darcy, to exclude the translocated newts from the Coed Darcy site and the remediation and construction activities. This sturdy plastic panel newt fence followed standard guidance (English Nature, 2001) and comprised 500 mm high plastic panels with a 50 mm overhang at the top and

300 mm buried underground; the fence was inspected monthly and repaired swiftly to maintain its integrity.

Capture and translocation

Capture and translocation of great crested newts was undertaken from 2009 to 2012 under licence from the Welsh Government; stopped from 2013 to 2015 to agree extensions to the area of the Receptor Site to support the numbers of captured great crested newts which were very much greater than expected from the newt surveys; resumed in 2016 under licence from NRW, the statutory nature conservation agency in Wales; and followed the standard guidance used in Britain (English Nature, 2001).

Capture of great crested newts relies on the movement of adult newts in the spring from terrestrial

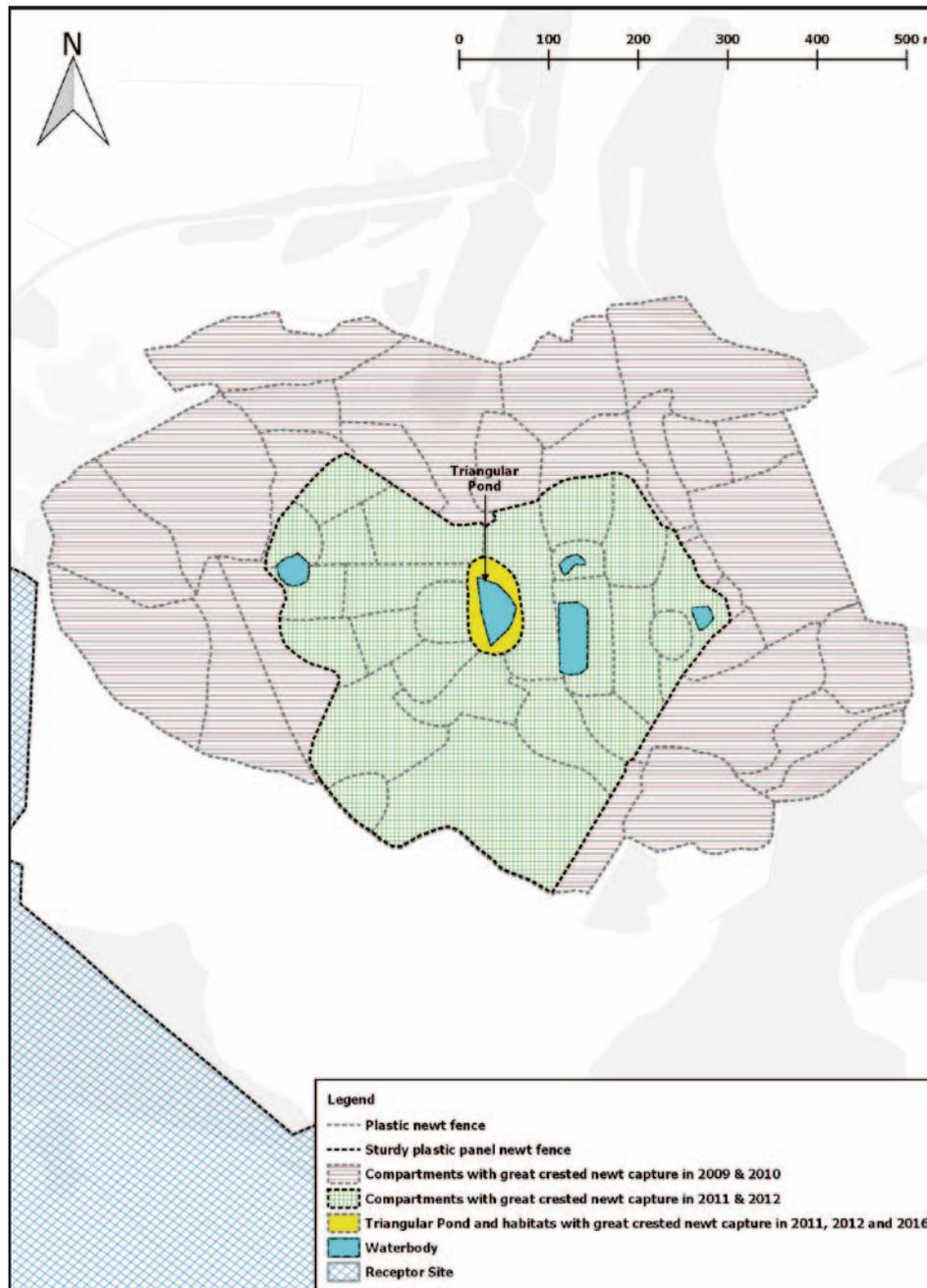


Figure 5. Arrangement of compartments for trapping great crested newts 2009-2016

habitats towards waterbodies to breed. At Coed Darcy, there was agreement with NRW that great crested newts would use suitable habitats within an integrated zone of 250 m around the five waterbodies containing great crested newts (the 'newt zone') (Fig. 1). The movements of the newts were intercepted by plastic newt fences that both completely enclosed the newt zone (47.1 ha) and divided this area into compartments (a total of 11 km of newt fences were installed over the whole of the capture programme) (Fig. 5). These plastic newt fences followed standard guidance (English Nature, 2001) and generally comprised 500 mm high plastic membrane with a 50 mm overhang at the top and with 300 mm plastic buried underground with 200 mm vertical depth and 100 mm horizontal underlap. A sturdy plastic panel newt fence surrounded the 'central compartment' (see below) and comprised 500 mm high plastic panels with a 50 mm overhang at the top and 300 mm buried underground. All the newt fences were inspected daily when the newt captures were taking place and swiftly repaired to maintain their integrity; these inspections took place monthly during the winter months, and during 2013 to 2015 when there was no capture programme.

Guided by these fences, the newts were caught in pitfall traps at 10 m intervals along the fences with an artificial refuge (a carpet tile approximately 0.5 m square) placed between each pair of pitfall traps (in locations where pitfall traps could not be used due to waterlogged or unsuitable ground, artificial refuges were placed at 5 m intervals). The pitfall traps were opened during the trapping season which lasted from March to October/November (depending on the weather). Newts were captured in the five waterbodies containing great crested newts using bottle traps set at 2 m intervals along the shorelines. The pitfall traps, artificial refuges and bottle traps were checked daily and great crested newts were transported to the Receptor Site (Figs. 1 & 3) together with other amphibians and reptiles. All life stages were translocated: larvae, juveniles with no external gills which had not reached sexual maturity (includes individuals after their first hibernation or 'immatures'), and adults.

Great crested newts captured in 2009 and 2010 could not be moved directly to the Receptor Site as the creation of additional ponds in the Receptor Site and their establishment as suitable habitats with vegetation was subject to planning, technical and construction delays. During these two years, a total of 430 great crested newts were captured in the 27 outer compartments of the 'newt zone' (30.4 ha) and were moved to a large 'central compartment' (16.7 ha) surrounded by a sturdy plastic panel newt fence (Fig. 5). This central compartment was subsequently divided into 24 compartments by plastic newt fences in early 2011. Capture of great crested newts in all these 24 compartments and translocation to the Receptor Site was undertaken in 2011 and 2012. Capture and translocation stopped in October 2012 with only one compartment not completely cleared of newts (0.58 ha comprising the Triangular Pond and surrounding terrestrial habitat). The capture programme stopped from 2013 to 2015 to agree extensions to the area of the Receptor Site to support the numbers of captured

great crested newts which were very much greater than expected from the newt surveys in 2008. Capture of great crested newts in the terrestrial and aquatic habitats in this final compartment restarted in March 2016 and was completed in November 2016.

Receptor Site monitoring

Annual monitoring (April to June) of the great crested newt population in the waterbodies in the Receptor Site and the condition of these waterbodies started in 2013. Between 2013 and 2015, monitoring was undertaken on the waterbodies that had been created and that were subject to habitat management. Monitoring was extended in 2016 and 2017 to include other waterbodies that were present in the Receptor Site. Monitoring in 2017 included Waterbody E (Fig. 3) that was created in 2015 and included within the newt fence around the Receptor Site in 2016.

Bottle trapping and torch counts were used to determine the population size class of the newts in the majority of the waterbodies following the standard guidance of six separate visits between mid-March and mid-June (English Nature, 2001). On occasion, lack of safe access to parts of the shoreline or shallow water levels precluded these techniques and the waterbody was monitored using netting and/or egg searches. Unsafe access and low water levels at Freeman's Bund and the most northerly of the Western Margin Ponds in 2017 resulted in monitoring using the environmental DNA (eDNA) methodology (Biggs et al., 2014). This involved a single visit on 4 July to collect 20 water samples which were sent for analysis (this date is just outside the recommended range of mid April to June for eDNA surveys).

The overall condition of the waterbodies was assessed using the Habitat Suitability Index (Oldham et al., 2000). The pH of the water bodies was measured in the field with a portable pH meter. The water bodies were inspected for the presence of plants suitable for egg-laying by newts, invasive non-native plants e.g. New Zealand pigmyweed (*Crassula helmsii*), fish and waterfowl. The shading of the waterbodies by adjacent trees and scrub and the condition of the artificial refuges/hibernation sites were assessed visually.

Deposition of data with local record centres

The data on great crested newts obtained from the surveys of waterbodies at Coed Darcy in 2008, the translocation programme, and the monitoring of the Receptor Site from 2013 onwards has been sent to the South East Wales Biodiversity Records Centre (SEWBReC) and will be sent to Cofnod which is the local environmental records centre for North Wales that hosts the data for the online great crested newt monitoring scheme for Wales (<https://www.cofnod.org.uk/LinkInfo?ID=7>).

Table 1. Great crested newts captured in 2011, 2012 and 2016 for translocation to the Receptor Site

| Year | Terrestrial habitats | | Waterbodies | | | Total |
|-------|----------------------|-----------|-------------|-----------|--------|-------|
| | Adults | Juveniles | Adults | Juveniles | Larvae | |
| 2011 | 587 | 1,165 | 1,369 | 280 | 121 | 3,522 |
| 2012 | 517 | 1,796 | n/a | n/a | n/a | 2,313 |
| 2016 | 640 | 1,040 | 1,027 | 9 | 990 | 3,706 |
| Total | 1,744 | 4,001 | 2,396 | 289 | 1,111 | 9,541 |

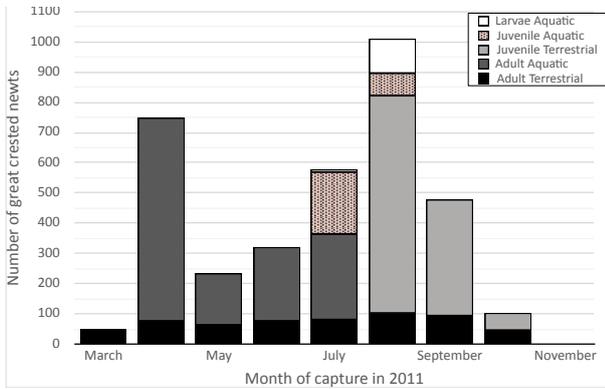


Figure 6. Seasonality of captures of great crested newts in 2011

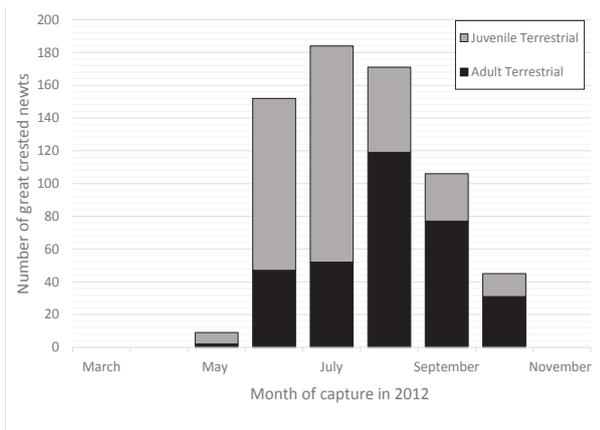


Figure 7. Seasonality of captures of great crested newts in 2012 (no waterbodies were involved)

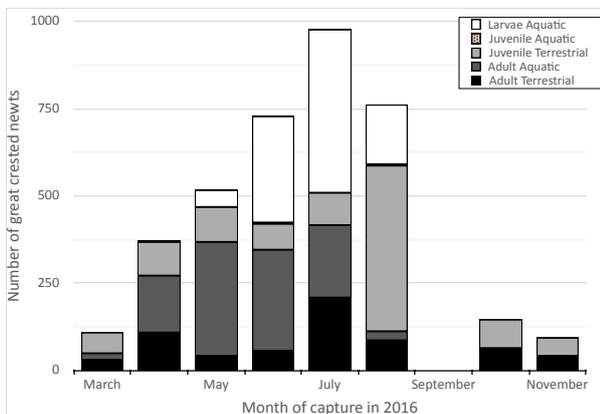


Figure 8. Seasonality of captures of great crested newts in 2016

RESULTS

Capture and translocation

Overall, 9,541 great crested newts were captured and translocated to the Receptor Site (4,140 adults, 4,290 juveniles, 1,111 larvae) (Table 1) as well as 4,094 palmate newts (*Lissotriton helveticus*), 116 smooth newts (*L. vulgaris*), 817 common frogs (*Rana temporaria*), 807 common toads (*Bufo bufo*), 82 common lizards (*Zootoca vivipara*) and 56 grass snakes (*Natrix natrix*).

Figures 6, 7 and 8 show the seasonality of the different life stages of the great crested newts captured in terrestrial and aquatic habitats in 2011 (24 compartments, five waterbodies including the Triangular Pond, 16.7 ha), in 2012 (26 compartments, no waterbodies, 15.9 ha) and 2016 (one compartment with the Triangular Pond, 0.58 ha).

Between April and July in 2011 and 2016, adult great crested newts were predominantly captured in the waterbodies. Capture of adult newts in waterbodies had almost ceased by August in both 2011 and 2016 as adults left the waterbodies to find suitable terrestrial habitat for foraging and hibernation. In terrestrial habitats, the number of adults captured each month remained relatively constant throughout 2011. There was more variation between monthly captures of terrestrial adults in 2012 and 2016 with peak numbers recorded in August and September in 2012 and in July in 2016.

Juvenile activity in the five waterbodies was recorded in July and August in 2011 (280 juveniles captured) but only nine juveniles were captured in the Triangular Pond in 2016 and these were captured between April and August. Terrestrial juvenile numbers peaked during August in 2011 and 2016, and in June and July in 2012, as individual newts completed metamorphosis and emerged from waterbodies to find suitable terrestrial habitat for foraging and hibernation.

Larvae were captured in July and August in 2011 in the five waterbodies and from May to August in 2016 in the Triangular Pond. Larvae are likely to have completed metamorphosis by late August and no larvae were captured between September and November.

Great crested newts were captured in the pitfall traps and under the carpet tiles placed adjacent to the newt fences and there was no noticeable pattern to the captures. However, there was one particular 20 m section of the newt fence between the Triangular Pond compartment and the adjacent compartment to the east where hundreds of great crested newts (mainly adults with some juveniles) were captured in 2011, 2012 and 2016. Peak numbers were captured along this particular section of newt fence during March to May in the traps in the compartment to the east of the Triangular Pond and during July to September in the traps in the compartment containing the Triangular Pond and associated terrestrial habitat.

Dead great crested newts were found along this one section on both sides of the newt fence. The dead newts had a hole in their abdomen, the abdominal organs were exposed and parts may have been removed; some dead newts were in several separate parts. It was assumed that the injuries were caused by a predator, probably a

bird. This first occurred in March and April 2011 (monthly totals of 102 and 11 newts respectively). Bird scarers (compressed gas air cannon) were used and compact discs (CDs) were hung on branches to discourage the birds and additional artificial refuges (carpet tiles) were placed side by side along both sides of the newt fence to avoid incidental death and injury. Dead great crested newts were recorded in subsequent years along both sides of this section of newt fence: seven in May 2012, 14 in July, 240 in August and five in September; two in March 2013; 62 in March 2014; and none in 2015 or 2016. There was no capture programme for great crested newts in 2013, 2014 and 2015 and the newts were found during the monthly inspections of the newt fences undertaken to maintain their integrity. Two dead palmate newts were found at this location in 2012 with holes in their abdomens.

Receptor Site monitoring

Monitoring was constrained on occasion by a lack of safe access to parts of the shoreline of a waterbody or by shallow water levels precluding the use of bottle traps. The pH of the water in the various waterbodies lies in the range 5.5 to 7.5. The results from the annual monitoring of the Habitat Suitability Index of the waterbodies from 2013 to 2017 are set out in Table 2. The target for this parameter was excellent (>0.8) or good (0.70-0.79). A score below 0.70 in one year is generally followed by a higher score in the next year because of habitat improvement works resulting from the annual monitoring.

The maximum adult count in the various waterbodies and the presence of eggs (indicating a breeding population) from 2013 to 2017 data are set out in Table 3. Peak counts of adult great crested newts can be obtained where there is regular interchange of newts between

waterbodies by summing the counts across all the waterbodies on the same visit. The peak counts of 153 in 2013, 95 in 2016 and 64 in 2017 were obtained following the standard methodology of six surveys between mid-March and mid-June (English Nature 2001). The peak counts of 49 in 2014 and 15 in 2015 are lower than the other three years, probably because the surveys in 2014 were restricted to two visits rather than the standard six surveys and the surveys in 2015 started in mid-May rather than mid-March. Eggs and larvae have been recorded in the new ponds and scrapes indicating that these created habitats provide favourable breeding conditions. Adult great crested newts were recorded in 2016 and 2017 in an area that had not been part of the original monitoring surveys (Waterbody B). In 2017, adult newts and eggs were recorded breeding in Waterbody E that was created in 2015 and integrated with the Receptor Site in 2016. Great crested newts were demonstrated to be present in the Western Margin Ponds and Freeman's Bund in 2017 using the eDNA methodology.

DISCUSSION

The original great crested newt population

Great crested newts were recorded at Coed Darcy in 2001 in the Triangular Pond at the lower end of the 'medium' population size class, in 2003 in the Triangular Pond as a 'small' population in 2003, and in 2008 in the Triangular Pond and four associated waterbodies as a metapopulation at the lower end of the 'medium' size class. This species was not recorded in 2008 in the other waterbodies on the Coed Darcy site nor in waterbodies within 500 m of the site boundary.

Great crested newts are usually considered to use terrestrial habitats up to 500 m from a breeding pond and long-distance migrations (> 1 km) are rare (English

Table 2. Habitat Suitability Index (HSI) for the waterbodies in the Receptor Site 2013-2017 (ND- not determined).

| Waterbody | Habitat Suitability Index ¹ | | | | |
|----------------------|--|------|------|------|------|
| | 2013 | 2014 | 2015 | 2016 | 2017 |
| Pond 1 | 0.77 | ND | 0.77 | 0.75 | 0.76 |
| Pond 2 | 0.87 | ND | 0.74 | 0.89 | 0.87 |
| Pond 3 | 0.80 | ND | 0.74 | 0.68 | 0.73 |
| Pond 4 | 0.87 | ND | 0.72 | 0.83 | 0.78 |
| The Scrapes | ND | ND | ND | 0.62 | 0.76 |
| Waterbody 29 | 0.84 | ND | 0.64 | 0.74 | 0.83 |
| Freeman's Bund | Not monitored between 2013-2015 ² | | | 0.83 | 0.75 |
| Waterbody A | Not monitored between 2013-2015 ² | | | 0.41 | 0.45 |
| Waterbody B | Not monitored between 2013-2015 ² | | | 0.44 | 0.51 |
| Waterbody C | Not monitored between 2013-2015 ² | | | 0.62 | 0.57 |
| Western Margin Ponds | 0.70 | 0.70 | 0.48 | 0.70 | 0.85 |
| Waterbody D | Surveyed as part of 'The Scrapes' 2013-2016 ³ | | | | 0.65 |
| Waterbody E | Created in 2015 and added to the Receptor Site in 2016 | | | | 0.81 |
| Small Pond | Surveyed as part of 'The Scrapes' 2013-2016 ³ | | | | 0.66 |

¹Habitat Suitability Index (Oldham et al., 2000): Excellent (HSI >0.8), Good (HSI 0.7-0.79), Average (HSI 0.6-0.69), Below Average (0.5-0.59), Poor (HSI <0.5).

²Monitoring of these waterbodies was undertaken in 2016 and 2017 to show how great crested newts are utilising the existing waterbodies within the Receptor Site.

³Waterbody D and Small Pond were surveyed as part of 'the Scrapes' between 2013-2016. The 2017 results from these ponds have been separated to show how great crested newts are utilising the individual ponds created within the Receptor Site.

Table 3. Great crested newt counts, population size and presence of eggs in the waterbodies in the Receptor Site 2013 – 2017 (NS - not surveyed).

| Waterbody | 2013 | | 2014 | | 2015 | | 2016 | | 2017 | |
|--|--|------|----------------------------|------|----------------------------|------|----------------------------|------|----------------------------|------|
| | Max. adult count ¹ | Eggs | Max. adult count | Eggs |
| Pond 1 | 25 | ✓ | 3 | ✓ | 2 | ✓ | 11 | ✓ | 10 | ✓ |
| Pond 2 | 55 | ✓ | 6 | X | 3 | ✓ | 25 | ✓ | 27 | ✓ |
| Pond 3 | 39 | ✓ | 3 | ✓ | 2 | ✓ | 10 | ✓ | 8 | ✓ |
| Pond 4 | 41 | ✓ | 35 | ✓ | 3 | ✓ | 55 | ✓ | 27 | ✓ |
| The Scrapes | 1 | X | 0 ² | X | 0 ² | X | 6 | ✓ | 7 | X |
| Waterbody 29 | 0 | X | 3 | X | 0 | X | 0 | X | NS ⁷ | X |
| Freeman's Bund | Not monitored between 2013-2015 ⁵ | | | | | | 0 | X | 0 ⁸ | X |
| Waterbody A | Not monitored between 2013-2015 ⁵ | | | | | | 0 | X | NS | NS |
| Waterbody B | Not monitored between 2013-2015 ⁵ | | | | | | 1 | X | 3 | X |
| Waterbody C | Not monitored between 2013-2015 ⁵ | | | | | | 0 | X | NS | NS |
| Western Margin Ponds | 1 | X | 0 | X | 0 | X | 0 | X | 0 ⁸ | X |
| Waterbody D | Surveyed as part of 'The Scrapes' 2013-2016 ⁶ | | | | | | | | 1 | X |
| Waterbody E | Created in 2015 and added to the Receptor Site in 2016 | | | | | | | | 4 | ✓ |
| Small Pond | Surveyed as part of 'The Scrapes' 2013-2016 ⁶ | | | | | | | | 6 | X |
| Receptor Site Peak Adult Count ³ and Population Size Class ⁴ | 153 Large population | | 49 Medium population | | 15 Medium population | | 95 Medium population | | 64 Medium population | |

¹ The maximum adult count for a waterbody taken on any survey date between mid-March and mid-June.

² No adult newts but larvae present.

³ The peak adult count for the Receptor Site involving all the waterbodies on the same survey date between mid-March and mid-June (English Nature, 2001, section 5.8.3).

⁴ Small < 11, medium 11-100, large >100 (English Nature, 2001, section 5.8.3).

⁵ Monitoring of these waterbodies was undertaken in 2016 and 2017 to ascertain whether great crested newts were utilising the existing waterbodies within the Receptor Site.

⁶ Waterbody D and Small Pond were surveyed as part of 'The Scrapes' between 2013-2016. The 2017 results from these waterbodies have been separated to ascertain how great crested newts were utilising the waterbodies created within the Receptor Site.

⁷ No population monitoring surveys were undertaken. An eDNA survey was planned, but an adult great crested newt was observed in July 2017 and therefore the planned eDNA survey was not undertaken.

⁸ eDNA survey confirmed great crested newt presence.

Nature, 2001; Jehle et al., 2011) and the maximum distance recorded is 1.6 km (Haubrock & Altrichter, 2016). The South East Wales Biodiversity Records Centre has two records from 2013 from Swansea, one approximately 4.5 km to the south-west and the other 5 km to the north-west of the Coed Darcy site, and a record from 2016 from Neath approximately 6.7 km to the north-east. Natural colonisation of the Triangular Pond by terrestrial migration can be discounted unless loss of intermediate ponds with breeding newts is postulated. The risk of extinction of such an isolated population of great crested newts is relatively high as demonstrated through population modelling by Griffiths & Williams (2001). The persistence of this population at Coed Darcy is notable.

The translocated population of great crested newts

This translocated population of great crested newts at Coed Darcy (9,541 newts of all life stages) is one of the largest translocations and one of the largest recorded populations in Britain. Around 24,000 adult and 5,000 juvenile great crested newts were translocated at Orton brickpits near Peterborough in Cambridgeshire to the Hampton reserve (Herpetofauna Consultants International, 2007); now part of the Orton Pit Special Area of Conservation (SAC), this is the largest known

population in the UK (Jehle et al., 2011; Joint Nature Conservation Committee undated). Around 6,000 great crested newts were translocated in three separate operations at Standard claypit, Brookhill claypit and Lane End claypit (now part of Deeside and Buckley Newt Sites SAC) (Natural Resources Wales, 2008; Liz Howe, NRW, pers. comm., 13 March 2017).

The pattern of captures of the three life stages in terrestrial and aquatic habitats between March and November (Figs. 6, 7 & 8) accords with the seasonality of this species (English Nature, 2001; Langton et al., 2001; Jehle et al., 2011). The capture of large numbers of juveniles and larvae (56.6 % of the translocated population) contributed to a final translocated population that was at least an order of magnitude larger than the population estimated in 2008 from the survey data. Capture and translocation schemes should complete the capture of newts by the end of June to minimise the numbers of juveniles and larvae whose numbers cannot be predicted but which count to the limits specified in the licences required for such schemes.

Adult great crested newts with some juveniles were captured in large numbers in all three trapping years (2011, 2012, 2016) along a short section of the newt fence between the compartment with the Triangular Pond and the adjacent compartment to the east. The

data suggest that the newts were moving towards the Triangular Pond from the adjacent compartment to the east during the breeding season (March to May); after the breeding season, newts were captured between July and September moving from the Triangular Pond towards suitable terrestrial habitat in the adjacent compartment. The habitat on both sides of the newt fence at this particular short section comprised woodland, dense bramble (*Rubus fruticosus* agg.) scrub, and piles of dead wood; there was more scrub and woodland in the compartment on the eastern side of the fence than in the compartment with the Triangular Pond. The habitats in the adjacent compartments to the north, south and west of the compartment with the Triangular Pond comprised rough grassland, ruderal vegetation and bramble scrub. Woodland is a preferred migratory habitat (Malmgren, 2002; Jehle et al., 2011, pages 53-54) and research has shown that juveniles (metamorphs) can detect cues, probably olfactory, left by both adults and juveniles and may be able to use these for orientation in relation to finding suitable terrestrial habitat (Hayward et al., 2000). Predicting such migration routes, augmenting the numbers of pitfall traps and artificial refuges, and confirming such predicted routes during a capture and translocation scheme may increase the effectiveness of such a scheme, help to reduce the time required for capturing the newts, and avoid predators killing newts in localised concentrations.

The daily trapping data indicated that the final compartment comprising the Triangular Pond and the surrounding terrestrial habitats had almost been cleared of great crested newts when the capture exercise was terminated in October 2012 because the Receptor Site had to be further extended to accommodate the numbers of captured newts which were substantially greater than expected from the 2008 survey data. The translocation was restarted in 2016 after additional terrestrial habitats were included in the Receptor Site together with the creation of a new waterbody. It is inferred that the newts captured in the compartment with the Triangular Pond during 2016 had developed from a very small residual population over the intervening three years. The density of 3,400 adult newts/ha using the terrestrial habitats (0.49 ha) around the Triangular Pond calculated from the trapping results for this compartment in 2016 (1,667 adults) is more than twice the highest densities previously reported of 1,250 to 1,500 great crested newts/ha in deciduous woodland (Oldham, 1994; Latham et al., 1996). However, this density was derived from the residual population of great crested newts that was isolated for three years in a fenced compartment with good quality terrestrial and aquatic habitats but with no opportunities for dispersal to adjacent habitats. Such a very high density of newts was an artefact of the trapping programme. The greater numbers of larvae captured in the Triangular Pond in 2016 (990 larvae) than in 2011 (121 larvae) with comparable terrestrial and aquatic trapping effort in both years is likely to be the result of this artificially large population.

The high mortality rates of juvenile great crested newts (80%) and larvae (95%) (Griffiths & Williams, 2001)

suggest that the number of adult newts translocated (4,140) may represent a more reliable estimate of the population in the Receptor Site. The population density of 180 adult newts/ha (370 newts of all life stages/ha) in the Receptor Site (26.3 ha) is comparable to those reported for good quality terrestrial habitats (Oldham, 1994; Latham et al., 1996; Langton et al., 2001, p.29; Box, 2017) and the general occupancy estimates of 250-350 newts/ha used for the translocation of a very large population of great crested newts at Hampton Reserve/Orton Pit (Herpetofauna Consultants International, 2007).

The great crested newt metapopulation was originally estimated as being between 50 and 750 great crested newts based on a cumulative peak count in 2008 of 15 adult newts summed across all waterbodies surveyed on the same survey date and the assumption that population surveys give estimates of between 2% and 30% of the actual population size (English Nature, 2001, section 5.8.3). The translocated population was much larger for reasons that may include:

- a) The newt surveys in 2001, 2003 and 2008 were limited by various factors: differences in the numbers of surveys done in the survey year and differences in the survey methods; some waterbodies were too shallow to use bottle traps; and because scrub along the shorelines of some waterbodies was so dense that access to the waterline was not possible (e.g. only 10% of the shoreline of the Triangular Pond, which was the key breeding site, was accessible in the 2008 surveys);
- b) Large piles of rubble colonised by scrub that were adjacent to the Triangular Pond and the waterbodies with breeding great crested newts would have provided ideal foraging habitat and refuges, thus greatly increasing the ability of the habitats to support newts;
- c) The waterbodies with great crested newts were in the southern tank farm of the oil refinery that was spread over a large area that would have been generally undisturbed apart from dedicated access routes and footpaths (John Smith, BP & St. Modwen, pers. comm., 7 February 2017).

Redgrave (2009) reported a great crested newt translocation at a site in Hampshire in England where the results of a population class size estimate of the water bodies was 29 adult newts, but the resultant translocation over 5 years involved 1,492 adult newts and 5,289 juvenile newts. The issues resulting from the lack of appropriate surveys prior to a translocation of great crested newts in Sweden are described by Gustafson et al. (2016). Griffiths et al. (2015) provide a cogent overview of the influence of site-specific and survey-specific variables that can affect counts of great crested newts and suggest that population assessments may more reliably reflect species detectability than actual population status.

One key lesson learned from the Coed Darcy scheme is that detailed surveys and population estimates are essential in situations where access to pond margins is restricted (for example, by dense scrub) and where

terrestrial habitat suitability for great crested newts is high. It is necessary to remove dense scrub or cut access paths to the edge of the water in order to survey a waterbody effectively otherwise there will be uncertainty about the size of the newt population because the survey was limited by access to the water. The use of Dewsbury traps (Dewsbury, 2011) in addition to the bottle traps used in the Triangular Pond in the capture programme in 2016 would have given even more certainty that all the newts had been captured before the destructive search of this waterbody.

The future for the great crested newt population

The current conservation status of the species in Wales is considered to be unfavourable declining; the exception is where appropriate long-term management is being implemented (Haysom et al., 2018). The long-term outcome of such compensation schemes is very dependent on the effectiveness of mechanisms to ensure site safeguard and routine habitat management (Edgar et al., 2005; Jehle et al., 2011; Lewis et al., 2017). The great crested newt monitoring and habitat management plan for Coed Darcy aims to maintain the nature conservation value and habitat suitability of the terrestrial habitats and aquatic features within the Receptor Site, and to promote the viability and longevity of the great crested newt population in the long-term. Annual monitoring and management of the great crested newt population and management of the habitats in the Receptor Site will continue up to and beyond 2032 (the period of the current management and monitoring plan) subject to discussions with NRW on the frequency of the population monitoring in the context of the monitoring results and the phased development of Coed Darcy. This monitoring informs the targeted habitat management in the Receptor Site and provides NRW with data for their reporting to the European Union in respect of the licence for the original translocation which permitted a derogation from the EC Habitats Directive (Council Directive 92/43/EEC). The importance of such long-term monitoring of amphibian translocations is emphasised by Germano & Bishop (2009).

Monitoring the population of great crested newts at the Receptor Site at Coed Darcy was constrained on occasion by a lack of safe access to parts of the shoreline of a waterbody or by shallow water levels precluding the use of bottle traps. The monitoring data from 2013 to 2017 (Table 3) demonstrate that great crested newts are moving around the Receptor Site colonising the new ponds and scrapes and are being recorded in small waterbodies in the wet woodland that were not part of the original monitoring scheme. Application of the eDNA method (Biggs et al., 2014), in addition to the annual population surveys on the waterbodies monitored since 2013, could enable a wide range of waterbodies in the Receptor Site to be sampled which would not be possible with existing standard population monitoring methodologies. This would facilitate the determination of which waterbodies across the whole of the Receptor Site are being used by great crested newts. Advances in relation to estimating population size from

eDNA surveys (Buxton et al., 2017) may provide a much cheaper and more effective technique for estimating the size of the population in the Receptor Site than standard methodologies.

Breeding has been observed within multiple ponds throughout the monitoring period, which suggests that the habitats within the Receptor Site provide appropriate conditions for great crested newts. The Habitat Suitability Index scores for the original and the created waterbodies in the Receptor Site generally meet the target of good or excellent (>0.7) (Table 2). Lower scores trigger habitat management that takes account of the individual factors from the suite used to derive the HSI (Latham, 2006). The challenge is to maintain, and improve as required, the aquatic habitats because the quality of the water bodies that form the breeding sites for great crested newts is a major factor in the size of a great crested newt population (Oldham et al., 2000).

Once the land on the Coed Darcy site that is within 250 m of all the waterbodies within the Receptor Site is built and fully developed, the sturdy plastic panel newt fence forming the boundary between the Receptor Site and Coed Darcy will be removed and great crested newts will be able to use the woodlands, the landscaped areas and open spaces, and the residential gardens. The Receptor Site will be linked to the Coed Darcy Wetlands (Figs. 1 & 3) whose function as a key site for great crested newts was originally identified in the planning application and the Environmental Statement submitted to Neath Port Talbot County Borough Council (Parsons Brinckerhoff, 2005, 2006) and is described in the Coed Darcy Masterplan Progress Report (St. Modwen, 2011, sections 6.18 & 6.19). The expectation is that the planned relationship between the Receptor Site, the Coed Darcy Wetlands and the Crymlyn Bog/Cors Crymlyn, an internationally important wetland (Fig. 1), will become a practical demonstration of the vision for nature conservation that requires large-scale habitat restoration and habitat creation as part of coherent and resilient ecological networks (Lawton et al., 2010).

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The effects of geography, habitat, and humans on the ecology and demography of the Gopher tortoise in the southern Lake Wales Ridge region of Florida

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A 35-year (1967–2002) demographic study was conducted on the gopher tortoise (*Gopherus polyphemus*) from two different habitats on Archbold Biological Station located on the southern end of the Lake Wales Ridge in south-central Florida. We found geographic, habitat, and human-mediated effects on several aspects of its biology. Our findings underscore the necessity of long-term demographic data to more accurately answer ecological questions concerning long-lived species, such as how the gopher tortoise detectably might be affected by habitat quality and human activities.

Keywords: Demography, ecology, gopher tortoise

INTRODUCTION

The gopher tortoise (*Gopherus polyphemus*) is a medium-sized inhabitant of sandy uplands of the southern parts of Louisiana, Mississippi, Alabama, Georgia, and South Carolina, extending southward through mainland Florida (Ernst & Lovich, 2009; Powell et al., 2016). Excessive loss and fragmentation of its habitat, coupled with its delayed maturity and human predation, has resulted in the decline of the species throughout its geographic range (McCoy & Mushinsky 1992; McCoy et al., 2006; USFWS, 2013). In 1978, the Gopher Tortoise Council was formed specifically to address conservation of this ancient and ecologically sensitive species. At that time, comprehensive demographic data on the species were uncommon.

In 1967, James N. Layne (JNL) initiated a long, uninterrupted field research programme on Archbold Biological Station (ABS) in Lake Placid, Highlands County, Florida. During the period 1967–2002, ecological, morphological, and population information was gathered on the gopher tortoise from two main sites in sandhill or scrub. The goal was to understand long-term demography of this species as it responded to its geographic location, habitat, and human activity. We provide 35 years of gopher tortoise life history data from ABS. Further, we compare our findings to those of others as they relate to geography, habitat, and human mediation.

STUDY AREA AND METHODS

The Archbold Biological Station, founded in 1941 by Richard Archbold, is a 2101 ha private reserve in Lake Placid, Highlands County, Florida. The station is located on the southern end of the Lake Wales Ridge whose habitats consist of southern ridge sandhills, sand pine scrub, rosemary scrub, scrubby flatwoods, swales, bayheads, seasonal ponds, and areas that are human-disturbed (see FNAI 2010 for descriptions of these habitats). From temperature data collected during 1952–2004, mean-minimum air temperature for January is 8.33° C, and mean-maximum air temperature for July is 34.05° C. From rainfall data collected during 1932–2004, mean annual rainfall is 136.4 cm (range = 69–195 cm).

The original station property, the East Section, was 431.94 ha. The eight tracts of the West Section were 1204.17 ha. The total area of this study was 1735.48 ha. Beginning August 1967, tortoises were actively captured by hand and individually marked in the field with notches onto the edges of marginal scutes. Tortoises were studied at both the East and West Sections. Survey effort was greatest during the first 20 years by JNL and assistants. At time of capture, individuals were sexed, body mass was recorded, plastron length (PL) and width and straight-line carapace length (CL) were measured in mm. Gular scutes were measured from their base to the notch in mm. Time and location were recorded at capture, as were feeding or reproductive behaviours. Interactions with other

animals were also noted.

We used a minimum convex polygon to calculate the home range of each individual. We examined a subset of scats defecated by captured animals under a dissecting scope for diet analysis. We used Cormack-Jolly-Seber (CJS) open population models to estimate survivorship (White & Burnham 1999). We measured condition using Fulton's K for analysis ($\text{weight}/\text{length}^3$) (Stevenson and Woods, 2006) and Duncan's Multiple Range Test. Sample statistics were calculated using Excel, 2016. Normality was determined using the Anderson-Darlington normality test in MiniTab 13.0 (MiniTab statistical package Inc. State College, Pennsylvania). Means values are followed by standard deviation, and statistical significance was recognized at $P < 0.05$.

RESULTS

Population demographics

Population structure

At the east and west sections, the percent of adults in the population increased over time from as few as 31.6 % up to 69.4 % (Tables 1–2). However, across all years, the percent of adults comprising each of the populations was less than 50 %, the lower value of which was a mere 31.6 % in the East Section (Table 1). The overwhelming numbers of juveniles at both sites was apparent when body size distributions were examined (Figs. 1–2). During the earliest few years of the study (1967–1970) in the East Section, 145 new individuals of known sex and status were captured along with an additional 40 tortoises of unknown status. Of the 145 tortoises, 39.3 % were adults and most of those were very young as evidenced by body size (Fig. 3). Conversely, juveniles ranged widely in size and age (Fig. 3), indicative of an emerging population.

Table 1. Number of first captures of gopher tortoises (*G. polyphemus*) as a percentage of the total during each time period at the East Section site of the Archbold Biological Station, Lake Placid, Florida

| Year | Male | Female | Juvenile | Adult |
|-----------|--------|--------|----------|--------|
| 1967–1979 | 27.0 % | 7.8 % | 65.2 % | 34.8 % |
| 1980–1990 | 26.9 % | 16.3 % | 56.8 % | 43.2 % |
| 1991–2001 | 42.8 % | 26.6 % | 30.6 % | 69.4 % |
| 2002 | 38.5 % | 61.5 % | 0.0 % | 100 % |
| 1967–2002 | 23.3 % | 8.5 % | 68.2 % | 31.8 % |

Table 2. Number of first captures of gopher tortoises (*G. polyphemus*) as a percentage of the total during each time period at the West Section site of the Archbold Biological Station, Lake Placid, Florida

| Year | Male | Female | Juvenile | Adult |
|-----------|--------|--------|----------|--------|
| 1969–1979 | 40.5 % | 8.3 % | 51.2 % | 48.8 % |
| 1980–1990 | 31.9 % | 12.8 % | 55.3 % | 44.7 % |
| 1991–1998 | 52.8 % | 13.2 % | 34.0 % | 66.0 % |
| 1969–1998 | 37.1 % | 8.0 % | 54.9 % | 45.1 % |

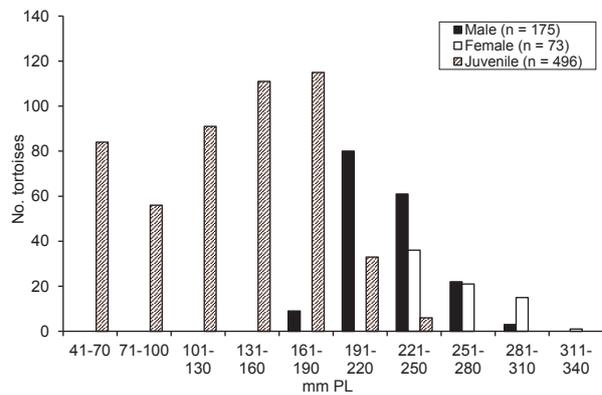


Figure 1. Body size distribution of first time captures of gopher tortoises (*G. polyphemus*) during the entire duration of the study in the East Section of the Archbold Biological Station, Lake Placid, Florida

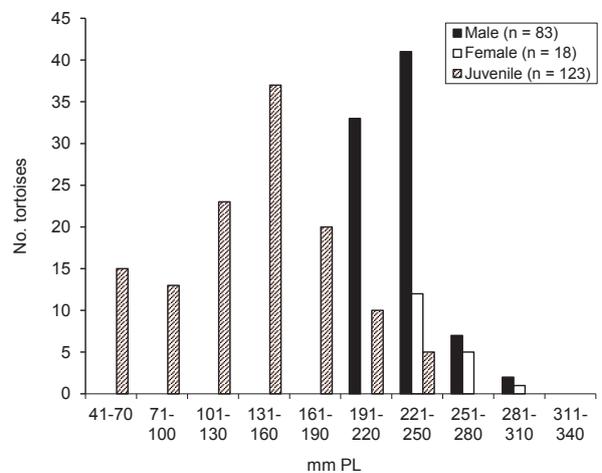


Figure 2. Body size distribution of first time captures of gopher tortoises (*G. polyphemus*) during the entire duration of the study in the West Section of the Archbold Biological Station, Lake Placid, Florida

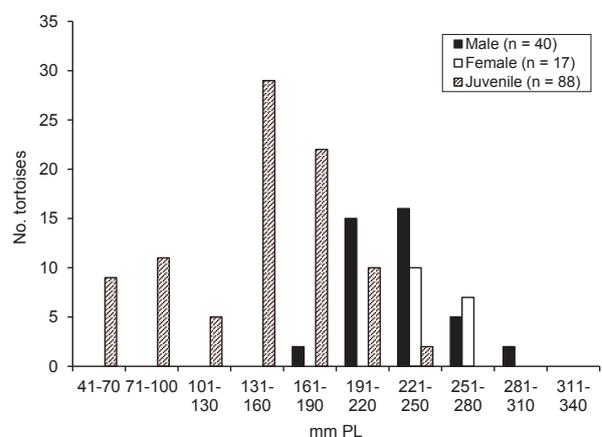


Figure 3. Body size (mm PL) distribution of first time captures of gopher tortoises (*G. polyphemus*) during 1967–1970 in the East Section of the Archbold Biological Station, Lake Placid, Florida

Sex ratio

During nearly all of the time periods and for combined time periods from both sites, adult males outnumbered adult females (Tables 3–4).

Population size

Overall in the East Section, males (Fig. 4) were more numerous than females (Fig. 5), and juveniles (Fig. 6) were more numerous than either sex at adult stage. Over time, numbers of males and females increased, whereas that of juveniles remained stable.

Table 3. Number of first captures of gopher tortoises (*G. polyphemus*) during each time period at the East Section site of the Archbold Biological Station, Lake Placid, Florida

| Year | Male | Female | Juvenile | Total | Unknown | Final Total |
|-----------|------|--------|----------|-------|---------|-------------|
| 1967–1979 | 103 | 30 | 249 | 382 | 51 | 433 |
| 1980–1990 | 104 | 63 | 220 | 387 | 35 | 422 |
| 1991–2001 | 74 | 46 | 53 | 173 | 13 | 186 |
| 2002 | 10 | 16 | 0 | 26 | 1 | 27 |
| 1967–2002 | 169 | 62 | 498 | 729 | 66 | 795 |

Table 4. Number of first captures of gopher tortoises (*G. polyphemus*) during each time period at the West Section site of the Archbold Biological Station, Lake Placid, Florida

| Year | Male | Female | Juvenile | Total | Unknown | Final Total |
|-----------|------|--------|----------|-------|---------|-------------|
| 1969–1979 | 34 | 7 | 43 | 84 | 3 | 87 |
| 1980–1990 | 30 | 12 | 52 | 94 | 4 | 98 |
| 1991–1998 | 48 | 12 | 31 | 91 | 10 | 101 |
| 1969–1998 | 83 | 18 | 123 | 224 | 9 | 233 |

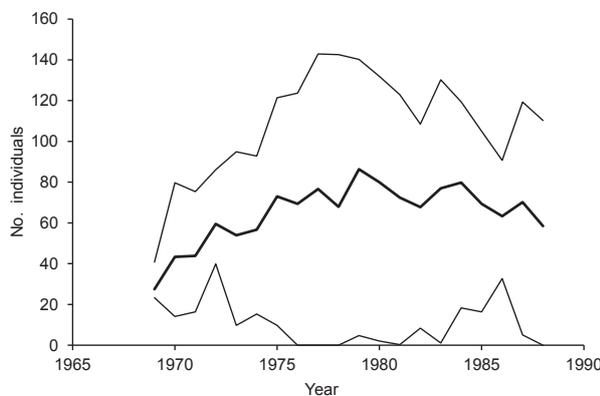


Figure 4. Population estimates of male gopher tortoises (*G. polyphemus*) during 1968–1988 in the East Section of the Archbold Biological Station, Lake Placid, Florida. Estimates are accompanied with upper and lower 95 % confidence levels.

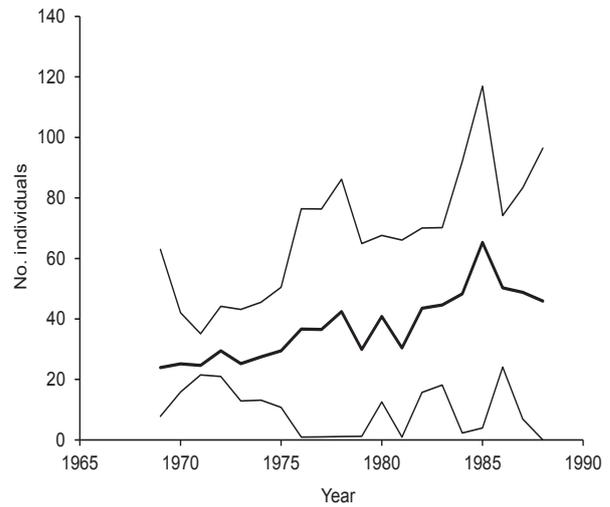


Figure 5. Population estimates of female gopher tortoises (*G. polyphemus*) during 1968–1988 in the East Section of the Archbold Biological Station, Lake Placid, Florida. Estimates are accompanied with upper and lower 95 % confidence levels.



Figure 6. Population estimates of juvenile gopher tortoises (*G. polyphemus*) during 1968–1988 in the East Section of the Archbold Biological Station, Lake Placid, Florida. Estimates are accompanied with upper and lower 95 % confidence levels.

Body size and condition

Minimum body size at sexual maturity

Based on dissections, the smallest male with enlarged testes relative to body size and active sperm in the vasa deferentia measured 186 mm PL. It was at this body size also that the relative length of the gulars was increasing from a minimum of about 12 % to a maximum of about 19 % of body size (Fig. 7). The smallest dissected female containing oviductal eggs was 225 mm PL. Using conversion formulas from regression equations (Fig. 8), we determined that the smallest sexually mature male measured 209.8 mm CL, and smallest sexually mature female measured 253.1 mm CL.

Mean body size at sexual maturity

The data distribution for plastron length (mm) was not

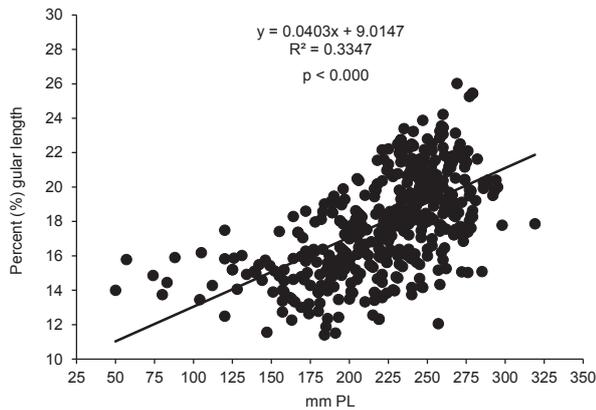


Figure 7. Relative length of the gular plate as a percentage of the plastron length on 415 male gopher tortoises (*G. polyphemus*) from the Archbold Biological Station, Lake Placid, Florida

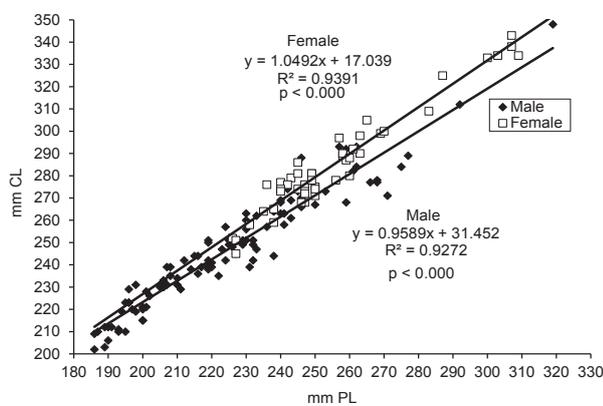


Figure 8. Relationship between body size in mm carapace length (CL) and body size in mm plastron length (PL) in male and female gopher tortoises (*G. polyphemus*) from the East Section of the Archbold Biological Station, Lake Placid, Florida

normally distributed ($A^2 = 1.283$, $P = 0.002$); therefore, we normalised the data using the normalise function within the MiniTab 13.0 statistical package (MiniTab, Inc. State College, Pennsylvania). The resulting normalised dataset was analysed with a General Linear Model with location and sex set as predictors. Interaction between sex and location was set as a third predictor. Male turtles (mean = 223.7 ± 22.0) were significantly smaller in CL than females (mean = 251.2 ± 19.4) across study sites ($P < 0.001$). Ignoring gender, CL did not differ between the eastern (mean = 229.7 ± 25.7) and western (mean = 231.24 ± 21.4) study sites ($P = 0.775$). However, PL may have been affected by an interaction between sex of a turtle and location ($F = 3.15$, $df = 1$, $P = 0.077$). Males from the eastern study site (mean = 221.6 ± 22.6 ; range = 186–302; $N = 163$) were similar in size to males (mean = 227.8 ± 20.3 ; range = 187–305; $N = 83$) from the western study site. However, females from the eastern (mean = 252.5 ± 19.3 ; range = 225–309; $N = 58$) study site were somewhat larger than those (mean = 246.9 ± 19.5 mm PL; range = 225–309; $N = 18$) from the western study

site. However, more data collection is needed to verify whether this is an artefact of smaller sample sizes among females.

Body size dimorphism

Body size dimorphism was weak in both populations. The ratio of mean male body size to mean female body size was high in both the East Section (0.88) and West Section (0.92).

Condition

Using Fulton's K for analysis (weight/length³) and Duncan's Multiple Range Test, we found significant effects of location ($P = 0.023$, East 2.52 > West 2.46), Sex ($P = 0.0001$, female 2.55, Juvenile 2.54 > male 2.46), but not season ($P = 0.0855$, Winter 2.56, Spring 2.52, Summer 2.509, Fall 2.501) or year ($P = 0.1350$) with respect to body condition of gopher tortoises. In effect, East Section tortoises fared better in condition, even if marginally so, than did their West Section counterparts. Although season did not affect condition of gopher tortoises, the difference among sex and age-class may have reflected the greater energy expenditure by males because of greater movements.

Growth and survivorship

Growth

Von Bertalanffy growth curves generated for both East and West Sections of the ABS assumed a hatchling size of 44 mm PL and minimum body sizes at sexual maturity of 186 mm PL for males and 225 mm PL for females. In the East Section, asymptotic growth was 260.7 mm PL for males (SE = 3.40; 95 % CI = 253.9–267.4) and 272.4 mm PL for females (SE = 4.65; 95 % CI = 263.2–281.6). The time necessary to reach sexual maturity was 7.39 years for males (95 % CI = 6.31–9.31) and 12.74 years for females (95 % CI = 10.08–17.10) (Fig. 9). In the West Section, asymptotic growth was 248.1 mm PL for males (SE = 5.07; 95 % CI = 237.7–258.5) and 257.3 mm PL for females (SE = 8.61; 95 % CI = 239.0–275.5). The time necessary to reach sexual maturity was 8.42 years for males (95 % CI = 5.88–13.45) and 10.27 years for females (5.39–31.51) (Fig. 10). It appears from our findings that the West Section tortoises were growing at rates that were similar to those of the East Section. We are cautious about this comparison in light of the smaller sample sizes in the West Section, as evidenced by the greater confidence intervals.

Survivorship

We estimated survivorship using CJS open population models. Best model estimated equal survival for adult males and females ($0.919 \pm SE 0.0079$). Juvenile survivorship was slightly lower than that of adults but still high ($0.826 \pm SE 0.014$).

Recapture rates

Recapture rates differed by sex, age, and time. Generally speaking, capture probability ranged from about 30–80 % for adults and 15–40 % for juveniles. No annual variation or sex effects were found in transition rates from juvenile

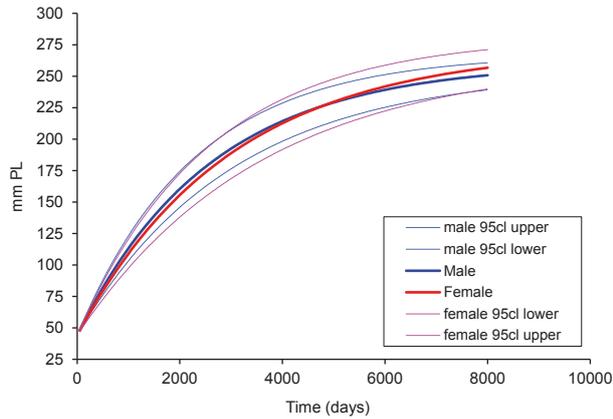


Figure 9. Von Bertalanffy growth curve estimated for gopher tortoises (*G. polyphemus*) of the East Section of the Archbold Biological Station, Lake Placid, Florida

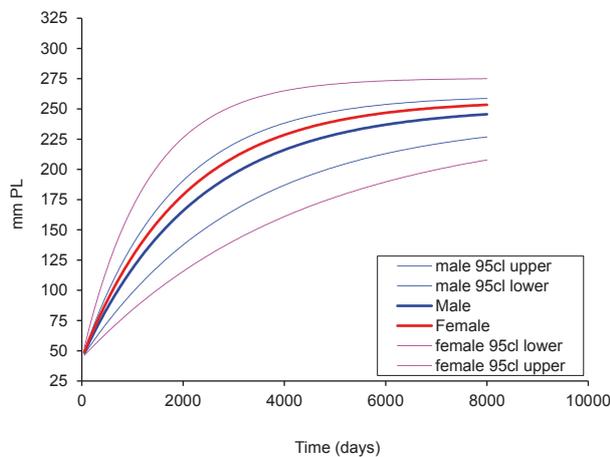


Figure 10. Von Bertalanffy growth curve estimated for gopher tortoises (*G. polyphemus*) of the West Section of the Archbold Biological Station, Lake Placid, Florida

to adult for either sex ($0.0399 \pm SE 0.005$). The 95 % Confidence Intervals were 0.9035 to 0.9345 for adult males and females and 0.7986 to 0.8534 for juveniles. The probability of a juvenile becoming an adult of either sex was about 4 % annually.

Activity

Seasonal

We found continuous activity out of the burrow by the ABS population with most activity occurring during April–August (Fig. 11). Among males, seasonal activity was greatest during April–August. Among females and juveniles, seasonal activity was greatest during May–August (Fig. 12). Indeed, among the marked animals, only 7.6 % of all captures occurred during the three coldest months of December–February.

Diel

On the ABS, the species was diurnal in its activity with activity centred around the warmest parts of the day, consistently avoiding late afternoon and morning during the winter (Fig. 13). Very few records associated

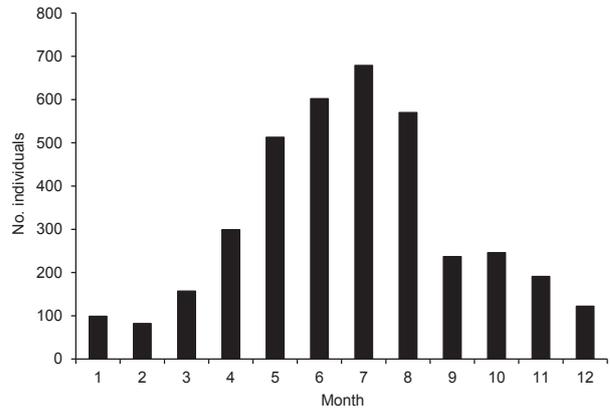


Figure 11. Combined records of all gopher tortoise (*G. polyphemus*) sightings ($n = 3,797$) for each month during 1967–2002 on the Archbold Biological Station, Lake Placid, Florida.

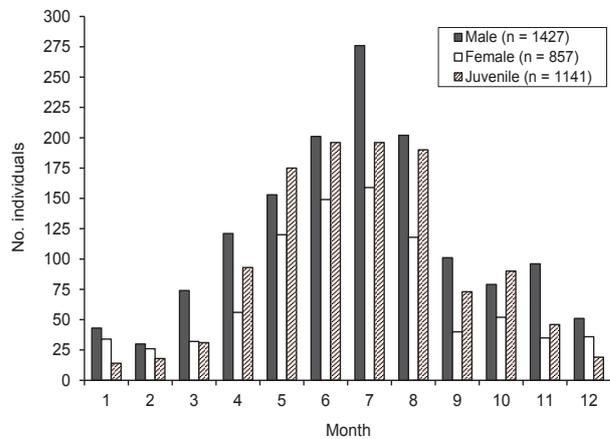


Figure 12. Combined records of all male, female, and juvenile gopher tortoise (*G. polyphemus*) from mark-recapture records for each month during 1967–2002 on the Archbold Biological Station, Lake Placid, Florida

with Figure 13 provided an estimation of cloud cover. Consequently, we cannot rule out the possibility that mixing cloudy-cool day with sunny-hot day records could be responsible for the observed peak in distribution of afternoon activity in summer. The range of active hours was greatest during the hottest months.

Movements

Distance moved from original capture and between captures

Movement data for gopher tortoises from the eastern study site (Table 5) were not distributed normally (Anderson-Darling: $A^2 = 16.59$, $P < 0.001$). Movements for adult male and female gopher tortoises from the eastern study site (Table 5) were not distributed normally (Anderson-Darling: $A^2 = 8.851$, $P < 0.001$). Adult gopher tortoises from the eastern site (mean = $1,498.4 \pm 1,499.2$ m; $N = 162$) moved more from site of original capture (two sample $t = 5.36$, $df = 173$, $P < 0.001$) than did juveniles (mean = $781.1 \pm 1,063.7$ m; $N = 89$). Adult male gopher tortoises from the eastern study site moved somewhat more from site of original capture than did females

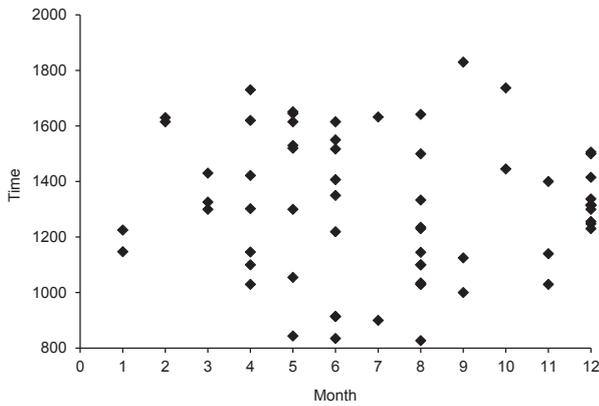


Figure 13. Combined records of all gopher tortoise (*G. polyphemus*) sightings (n = 64) for each hour during 1967–2002 on the Archbold Biological Station, Lake Placid, Florida

Table 5. Mean movements (m) by gopher tortoises (*G. polyphemus*) from the East Section of the Archbold Biological Station, Lake Placid, Florida. Means are followed by standard deviation.

| Category | Mean movement (m) from previous site of capture | Mean movement (m) from original site of capture |
|----------|---|---|
| Male | 1,552.5 ± 1,554.8; range = 40–8030; N = 99 | 1,645.5 ± 1,586.5; range = 40–8030; N = 99 |
| Female | 1,202.7 ± 1,223.5; range = 40–5740; N = 63 | 1,267.3 ± 1,330.2; range = 40–6020; N = 63 |
| Juvenile | 768.4.0 ± 1,099.4; range = 0–5000; N = 89 | 781.1 ± 1,063.7; range = 0–5000; N = 89 |

(Table 5) but not statistically so (two sample $t = 1.79$, $df = 133$, $P = 0.075$). From further analysis using Tukey’s pairwise comparison, we found that mature males did not move more than mature females (Tukey: -0.1145, 0.5925); but, juveniles moved significantly less than either adult males (Tukey: 0.4473, 1.0881) or females (Tukey: 0.1676, 0.8899). For males (Fig. 14), females (Fig. 15), and juveniles (Fig. 16), movements were greatest in distance and most numerous during April–October.

Distance moved from previous site of capture

Movements among gopher tortoises were not distributed normally (Anderson-Darling: $A^2 = 17.9$, $P < 0.001$). Movements from previous site of capture in the East Section (Table 5) among adults (mean = 1416.5 ± 1441.3 m; range = 40–8030) were significantly larger (two sample $t = 3.99$, $df = 133$, $P < 0.001$) than those of juveniles (mean = 768.4 ± 1099.4 m; range = 0–5000). Males, females, and juveniles dispersed differently (Table 5) since the previous encounter ($F = 8.20$, $df1 = 2$, $df2 = 250$, $P < 0.001$). Males moved significantly further (Tukey: 331, 1237) than did juveniles. Female movements were intermediate between those of males and juveniles and not significantly different from males (Tukey: -150, 850) or juveniles (Tukey: -77, 945). Generally, females moved less than males but more so than juveniles from site of last capture.

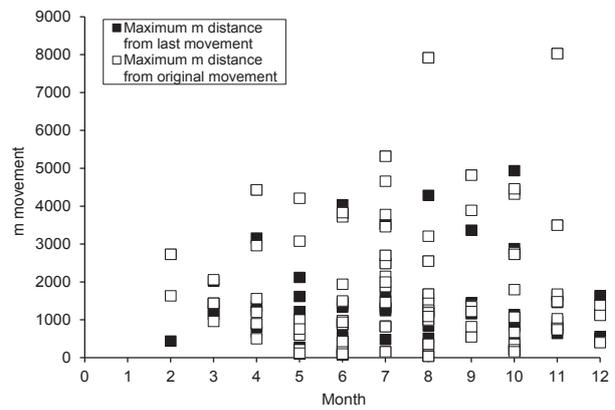


Figure 14. Monthly distance from original capture site and from last capture site for 99 male gopher tortoises (*G. polyphemus*) from the East Section of the Archbold Biological Station, Lake Placid, Florida

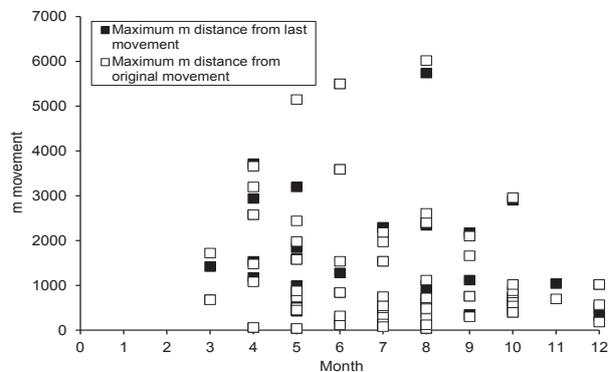


Figure 15. Monthly distance from original capture site and from last capture site for 63 female gopher tortoises (*G. polyphemus*) from the East Section of the Archbold Biological Station, Lake Placid, Florida

Home range size- The home range data for gopher tortoises were not normally distributed ($A^2 = 8.63$, $P < 0.001$). The home range data for mature male and female gopher tortoises were not normally distributed (Anderson-Darling: $A^2 = 7.883$, $P < 0.001$). The home range sizes of mature gopher tortoises (mean = 6.07 ± 7.0 ha; N = 102) were not significantly different in size (two-sample $t = 1.04$, $df = 188$, $P = 0.31$) than those of immature individuals (mean = 1.32 ± 6.7 ha; N = 6). The home ranges of mature males (6.3 ± 7.3 ha; range 0.07–36.5; N = 64) were not significantly different (two-sample $t = 1.04$, $df = 74$, $P = 0.304$) from those of mature females (5.6 ± 6.5 ha; 0.1–26.6; N = 38). Collectively, adults occupied larger home ranges than did juveniles ($F = 6.55$, $df = 1$, $P = 0.012$).

Habitat use

In the East Section, males, females, and juveniles were most often found in southern ridge sandhill, followed by scrubby flatwoods, and sand pine scrub (Table 6). More specifically, gopher tortoises were found more often in turkey oak phase sandhill than in hickory phase (Table 7). In scrubby

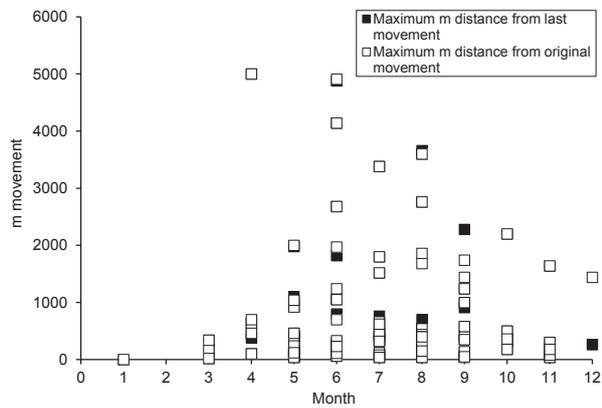


Figure 16. Monthly distance from original capture site and from last capture site for 81 juvenile gopher tortoises (*G. polyphemus*) from the East and West Sections of the Archbold Biological Station, Lake Placid, Florida

Table 6. Number of records of individuals for each general habitat type used by gopher tortoises (*G. polyphemus*) from the East Section of the Archbold Biological Station, Lake Placid, Florida

| Habitat | Male | Female | Juvenile | Total |
|-------------------------|------|--------|----------|-------|
| Bayhead | 1 | 0 | 2 | 3 |
| Flatwoods | 83 | 49 | 84 | 216 |
| Improved pasture | 1 | 0 | 0 | 1 |
| Ruderal | 84 | 56 | 49 | 189 |
| Sand pine scrub | 186 | 104 | 148 | 438 |
| Scrubby flatwoods | 236 | 148 | 228 | 612 |
| Seasonal Pond | 4 | 4 | 3 | 11 |
| Southern Ridge sandhill | 401 | 278 | 331 | 1010 |
| Total | 926 | 595 | 776 | 2297 |

flatwoods, gopher tortoises were found more often in live oak phase than in the inopina oak phase (Table 7). Of the remaining habitats, flatwoods and ruderal habitats were used extensively by gopher tortoises. Far and away, palmetto phase was the preferred flatwoods type, and old field was used most often by gopher tortoises in human-modified habitats (Tables 6–7).

Burrow dynamics

Tortoise use

Over the 24-year period, only three (1.6 %) of 192 active burrows initially detected were still active, the steep decline of which was evident the following year of the study (Fig. 17). Likewise, of 16 inactive burrows, only one (6.3 %) burrow subsequently became home to a gopher tortoise (Fig. 17), suggestive of little competition for burrows. The burrow widths co-varied significantly ($P < 0.000$) with the shell width of the largest resident at each of the three distances from the burrow entrance (Fig. 18).

Commensal vertebrate species

On the ABS, the following vertebrates were observed by

Table 7. Number of records of individuals for each specific habitat type used by gopher tortoises (*G. polyphemus*) from the East Section of the Archbold Biological Station, Lake Placid, Florida.

| Habitat | Male | Female | Juvenile | Total |
|--|-------|--------|----------|-------|
| Bayhead | 1 | 0 | 2 | 3 |
| Flatwoods-cutthroat grass phase | 4 | 4 | 2 | 10 |
| Flatwoods- gallberry phase | 9 | 1 | 13 | 23 |
| Flatwoods- palmetto phase | 70 | 44 | 69 | 183 |
| Improved pasture | 1 | 0 | 0 | 1 |
| Human-modified oldfield | 66 | 53 | 43 | 162 |
| Human-modified garden | 18 | 3 | 6 | 27 |
| Human-modified landscape | 28 | 24 | 37 | 89 |
| Sand pine scrub-mature oak phase | 186 | 104 | 148 | 438 |
| Scrubby flatwoods-live oak phase | 187 | 98 | 158 | 443 |
| Scrubby flatwoods-inopina oak phase | 49 | 50 | 70 | 169 |
| Seasonal pond | 4 | 4 | 3 | 11 |
| Southern Ridge sandhill-hickory phase | 160 | 93 | 160 | 413 |
| Southern Ridge sandhill-turkey oak phase | 241 | 185 | 171 | 597 |
| Total | 1,024 | 663 | 882 | 2,569 |

JNL entering or leaving gopher tortoise burrows: Florida gopher frog (*Lithobates capito aesopus*), southern black racer (*Coluber constrictor priapus*), eastern indigo snake (*Drymarchon couperi*), eastern coachwhip (*Masticophis flagellum flagellum*), Florida pine snake (*Pituophis melanoleucus mugitus*), Florida mouse (*Peromyscus floridanus*), cotton mouse (*Peromyscus gossypinus*).

Reproduction

Fertility

Males dissected in May (193 mm PL) and October (186 mm PL) had enlarged yellowish-coloured testes with abundant sperm in the ducts (Fig. 19), indicating fertility at least during May–October for which we have data (Fig. 19).

Nesting

On the ABS, a female with enlarged ova (32 mm) was found on 29 April 1977. Females with shelled eggs were found during May (N = 3), and nests were found in May (N = 1) and June (N = 2).

Clutch size

Five shelled eggs were recovered from a dissected 225 mm PL female. Two nests excavated in the field contained five and six eggs.

Annual clutch production

From a single dissected female, no evidence existed for the production of more than one clutch for the season.

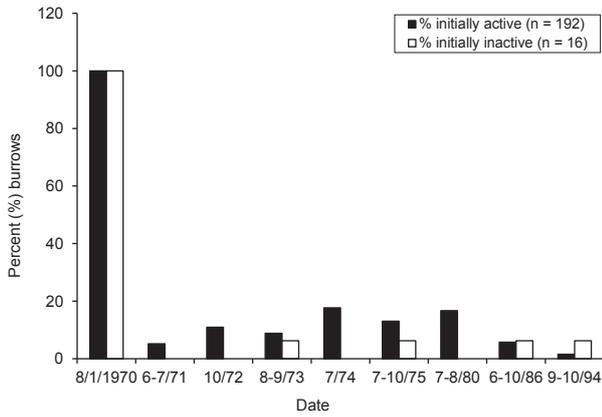


Figure 17. Temporal changes in the status of gopher tortoise (*G. polyphemus*) burrows on the Archbold Biological Station, Lake Placid, Florida

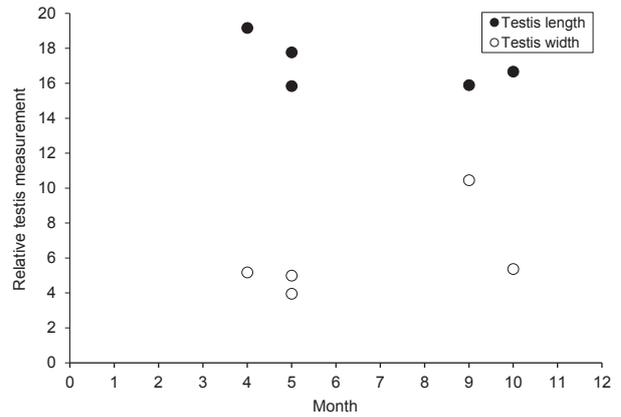


Figure 19. Monthly distribution of right testis lengths and widths as a percent of body length in five male gopher tortoises (*G. polyphemus*) on the Archbold Biological Station, Lake Placid, Florida

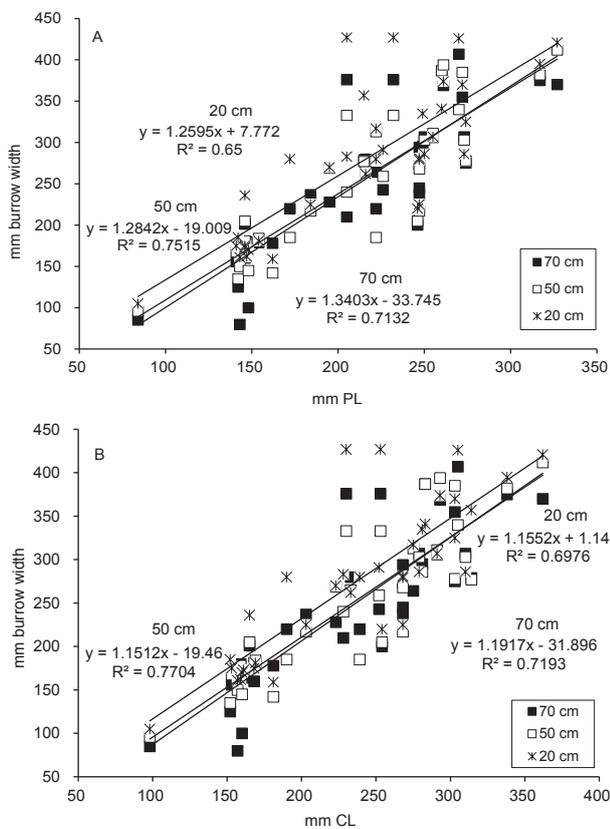


Figure 18. Relationship between burrow diameter at three different distances from the burrow entrance and body size in gopher tortoises (*G. polyphemus*) (n = 37) on the Archbold Biological Station, Lake Placid, Florida. **A** = plastron length, **B** = carapace length. All regression analyses were significant to $P < 0.000$.

Egg dimensions

Shelled egg dimensions for a five-egg clutch dissected from a 225 mm PL female found 12 June 1983 were 44.5 X 38.9 mm, 42.8 X 38.5 mm, 44.0 X 37.5 mm, 47.4 X 37.1 mm, 49.2 X 36.3 mm. Shelled egg dimensions for a six-egg clutch dug from the field 25 June 1979 were 44.5 X 43.2 mm, 46.3 X 41.0 mm, 46.0 X 42.7 mm, 45.4 X 44.0 mm, 48.2 X 42.1 mm, 46.6 X 42.8 mm.

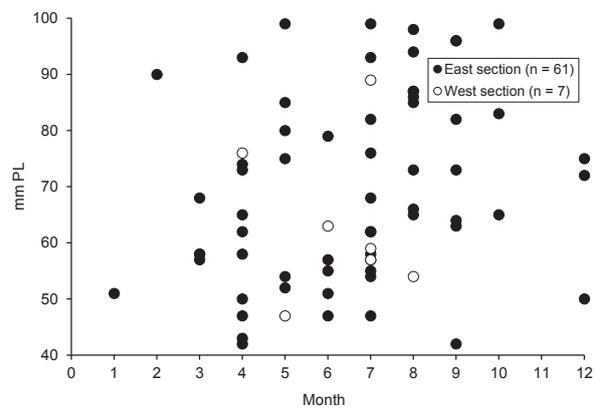


Figure 20. Monthly distribution body sizes of small gopher tortoises (*G. polyphemus*) captured in the East Section and West Section of the Archbold Biological Station, Lake Placid, Florida

Hatching season

On the ABS, the smallest individuals were found during April–September (Fig. 20).

Hatchling body size

On the ABS, the smallest individuals we found measured 42 mm PL (Fig. 20).

Diet

General

On the ABS, gopher tortoises were primarily, but not exclusively, herbivorous and ate a wide range of primarily plant items (Table 8). To that end, grasses (both native and landscape), saw palmetto, and pusley (*Richardia* spp.) were prominent in their diet as were various parts of pines and bromeliads (Table 8).

Coprophagy

Four instances of coprophagy by one female (263 mm PL) and three juveniles (163, 182, 192 mm PL) took place while the tortoises were being held for processing and had immediate access to the conspecific scats. In one of these instances, the scat was old and dry but was sought

Table 8. Diet as reported by numbers of tortoises associated with 203 records of 83 food categories from field observations, scat observations, and scat analysis of 127 gopher tortoises (*G. polyphemus*) on the Archbold Biological Station, Lake Placid, Florida

| Food item | No. tortoises with food item | Food item | No. tortoises with food item |
|---|------------------------------|---|------------------------------|
| <i>Richardia</i> spp. (includes <i>R. brasiliensis</i> and <i>R. scabra</i>) | 50 | Cyperaceae (<i>Fimbistylis</i> or <i>Bulbostylis</i>) | 1 |
| <i>Paspalum setaceum</i> | 33 | <i>Danthonia spicata</i> | 1 |
| <i>Tillandsia</i> spp. | 32 | <i>Dasypus novemcinctus</i> (rib bones) | 1 |
| <i>P. notatum</i> | 22 | Dermestidae | 1 |
| <i>Serenoa repens</i> | 19 | <i>Diachlorus ferrugatus</i> | 1 |
| Grass (unknown seeds and leaves) | 16 | <i>Drymarchon corais</i> (scutes) | 1 |
| Plant (unknown leaves, stems) | 15 | <i>Froelichia floridana</i> | 1 |
| <i>Pinus elliotii</i> (needle) | 13 | glass chunks | 1 |
| Euphorbiaceae (includes <i>Euphorbia maculata</i>) | 11 | <i>Gnaphalium falcatum</i> | 1 |
| <i>Vaccinium</i> spp. (includes leaf, capsule, seed, stem) | 11 | <i>Gnaphalium purpureus</i> | 1 |
| <i>Quercus</i> spp. (leaf) | 9 | <i>Heterotheca scabrella</i> | 1 |
| <i>Andropogon</i> (seed, stalk) | 8 | <i>Hydrocanthus</i> spp. | 1 |
| <i>Pinus</i> spp. (needle) | 8 | <i>Lactarius volenus</i> | 1 |
| <i>Gopherus polyphemus</i> feces | 5 | <i>Lycania michauxii</i> | 1 |
| <i>Opuntia</i> (seed) | 5 | <i>Myrica</i> sp. | 1 |
| Palmetto (seeds) | 5 | <i>Oldamlandia corymbosa</i> | 1 |
| Rock | 5 | <i>Opuntia</i> (spine) | 1 |
| Seed unknown | 5 | <i>Oxalis</i> spp. | 1 |
| <i>Selaginella</i> spp. | 5 | <i>Paspalum</i> spp. | 1 |
| <i>Diodella rigida</i> | 4 | <i>Persea</i> spp. (leaves) | 1 |
| <i>Pinus</i> spp. (cone petal) | 4 | <i>Polygonella fimbriata</i> | 1 |
| <i>Carya</i> sp. | 3 | <i>Quercus geminata</i> (leaf) | 1 |
| <i>Chapmannia floridana</i> | 3 | <i>Quercus myrtifolia</i> (cap) | 1 |
| Coleoptera (parts) | 3 | <i>Quercus virginianus</i> (whole acorns) | 1 |
| <i>Commelina erecta</i> var. <i>angustifolia</i> | 3 | Sand | 1 |
| <i>Diodella teres</i> | 3 | <i>Schinia rivulosa</i> | 1 |
| <i>Eremochloa ophiuroides</i> | 3 | <i>Serenoa repens</i> (berries) | 1 |
| <i>Lupinus</i> spp. (leaves, seeds) | 3 | <i>Serica frosti</i> | 1 |
| <i>Prosopis</i> | 3 | <i>Solanum</i> spp. | 1 |
| <i>Sabal etonia</i> (includes berries) | 3 | <i>Thrinax microcarpa</i> | 1 |
| <i>Schrankia</i> spp. | 3 | <i>Ximenia americana</i> | 1 |
| <i>Tragia urens</i> | 3 | | |
| Bird feathers | 2 | | |
| <i>Cenchrus</i> spp. | 2 | | |
| <i>Panicum</i> | 2 | | |
| <i>Parthenocissus quinquefolia</i> | 2 | | |
| <i>Sciurus carolinensis</i> (bones, fur, teeth) | 2 | | |
| <i>Setaria corrugata</i> | 2 | | |
| <i>Sida cordifolia</i> | 2 | | |
| <i>Smilax auriculata</i> | 2 | | |
| <i>Vitis</i> spp. | 2 | | |
| <i>Ampelopsis arbores</i> | 1 | | |
| <i>Asimina triloba</i> (seeds) | 1 | | |
| <i>Atrenius faggi</i> | 1 | | |
| <i>Berosus</i> | 1 | | |
| <i>Camaechrista fasciculata</i> | 1 | | |
| <i>Campsis radicans</i> | 1 | | |
| <i>Cassia chamaecrista</i> | 1 | | |
| <i>Chrysopsis graminifolia</i> | 1 | | |
| <i>Citrus</i> spp. (seed) | 1 | | |
| cocoon | 1 | | |

out and eaten by the 192 mm PL juvenile. In the field, a 281 mm PL female was observed eating an old dry conspecific scat. Besides potential nutritive value, we are unsure of the extent to which coprophagy helps or hinders subsequent germination of seeds already passed through once in its digestive system.

Predators

On the ABS, black bear (*Ursus americanus*) and Raccoon scat were found by JNL to have contained remnants of juvenile gopher tortoise.

Mortality factors

On the ABS, hog wire fence trapped and sometimes killed adults, and humans harvested active tortoises found alongside the road and inactive tortoises in burrows near the road.

DISCUSSION

Population Demographics

Population structure

The low percentage of adults in our two sites, particularly in the East Section, stood in sharp contrast to values reported elsewhere in Florida. For example, at a coastal location in south-western Florida, adults comprised 56.5 % of the captures (McLaughlin, 1990), and in 2013 at a south-eastern Florida coastal site in Jupiter, Florida, adults comprised 65 % of the censused population (Jon A. Moore, pers. comm.). Adult composition was high for both a pine flatwoods/hardwood hammock site (70 %) and a mostly pasture and some upland hammock site (77.1 %) in west-central Florida (Godley, 1989). At one sandhill and two modified habitats studied during 1981–1987 in northern Florida, adults comprised 40–54 % of the population (Diemer, 1992a). In a follow-up survey in 2009 (Diemer Berish et al., 2012), fewer juveniles were captured resulting in a different population structure. A population decline or difficulty in detectability of juveniles because of floristic changes was thought to be responsible for fewer captures of juveniles in 2009 (Diemer Berish et al., 2012). In north-central Florida adults comprised 80.6 % of populations (Smith, 1995). We are unsure to what extent the duration of this study affected the estimate of adult composition, whereby a long period of time of search would offset the difficulty of detecting small, inconspicuous members of the population. Compared to these other studies, habitat did not seem to control the composition of adults in the population. At least for the East Section, which borders a road and was also bisected partially by a road, we wonder if harvesting pressure on adults, particularly evident in the early years, resulted in more space for juvenile animals. To that end, the temporal trend in adult composition was to increase, such that by 1999, the adult composition (Table 1) was on par with that of other studies (op. cit.). In contrast, the West Section population was buffered from the road by unburned, low quality habitat thereby providing more, even if not complete, protection to a comparatively smaller population from harvesting. Consequently, the earliest values for adult composition were higher than those of the East Section, and by contemporary times, the adult composition was likewise on par with others (Table 2).

Sex ratio

At both of our sites, males tended to greatly outnumber females. In light of the intensive search effort during the early years of this study, the duration of this study, and the corroborative sex ratio values at different time intervals, we believe the male-biased sex ratio on the ABS to be affirmed. This finding was in keeping with many, but not all, other populations. For example, males outnumbered females in south-eastern Florida (Fucigna & Nickerson, 1989). The same was true at one sandhill and two modified habitats in northern Florida during 1981–1987 (1.00:1.31) (Diemer, 1992a) but differed (1.00:0.73) in a follow-up survey in 2009 (Diemer Berish et al., 2012). In a west-central Florida pine flatwoods/

hardwood hammock site, males outnumbered females, but were outnumbered by females at another site comprised primarily of pasture and secondarily by upland hammock (Godley, 1989). In west-central Florida (Linley, 1986) and in north-central Florida (Smith, 1995), the sex ratio was even. In coastal south-western Florida, the overall sex ratio was even but was female-biased at one site (McLaughlin, 1990). In Jupiter, Florida, females dominated slightly (0.88:1.00) in a 2013 census (Sano, 2014).

Habitat preference

Based on counts of animals and our model, findings on the ABS conformed to the findings that the gopher tortoise preferred grasslands or grassland analogues (Ashton & Ashton, 2004, 2008), such as the sandhill of the east section more than the eastern desert scrub of the west section on the ABS.

Population size

Population densities on the ABS were low, perhaps because of the poor quality of habitat, especially in the scrub of the west section. In the east section with 431.94 ha of living space, densities scarcely reached one tortoise/ha. Elsewhere in Florida, population densities averaged 16.7 tortoises/ha (range = 4.2–24.9 tortoises/ha) in four south-western Florida sites (McLaughlin, 1990).

On the ABS, adults could use more than one burrow (Douglass & Layne, 1978), tortoise body size and burrow widths were strongly correlated (Martin & Layne, 1987), and the burrows excavated by juveniles were shorter than those of adults and the widths of the burrows reflected the size of the juvenile (Meshaka & Layne, 2015).

Numbers of burrows provided a different measure of abundance. For example, burrow counts yielded a population estimate of 11.3 tortoises/ha in 1979 on Cape Sable (Kushlan & Mazzotti, 1982). Surveys on Cape Sable by McCoy & Mushinsky (1992) showed no changes during 1978–1979 or 1987–1988. Follow-up surveys in 2001 (Waddle et al., 2006) detected a sharp decline in estimated density of active burrows from 14.5/ha in 1979 to 4.4/ha in 2001 although the overall number of burrows remained relatively stable.

Mushinsky & McCoy (1994) noted that although population densities as estimated by number of burrows from many sites in Florida were less than 10 tortoises/ha, densities of two sites exceeded 30 tortoises/ha. Small parcels of suitable quality can support large populations. For example, more than 40 tortoises were found on a 1.21 ha coastal strand site near Vero Beach, Florida, indicating a population density of 27.5 tortoises/ha (Jon A. Moore, pers. comm.).

In Florida, a strong relationship was detected between numbers of active plus inactive burrows and area for both mainland and island sites, but density of gopher tortoises decreased as habitat increased on mainland but not islands perhaps because insular populations were forced to make do with what was available instead of dispersing to optimal habitat (Mushinsky & McCoy, 1994). Interestingly, 65 burrows/ha were calculated for a 1.21 ha coastal strand site in Vero, Beach, Florida

(Jon A. Moore, pers. comm.). In south-central Florida, densities of active burrows were lower in scrub (0.6/ha) and flatwoods/plantations (0.21/ha) (Castellón et al., 2012). Habitat quality varied with up to 2.7 tortoises/ha in disturbed habitat in east-central Florida (Breininger et al., 1994) to 7.6 tortoises/ha in northern Florida pine-oak habitat (Diemer, 1989). In north-central Florida, mean burrow densities for two sandhills were 2.4 burrows/ha and 10.6 burrows/ha, and mean densities for two old fields were 6.0 burrows/ha and 7.6 burrows/ha (Smith, 1995). In South Carolina, 1.8 tortoises/ha inhabited pine-turkey oak and 0.4 tortoises/ha were found in turkey oak-hawthorn (Wright, 1982).

Body size at sexual maturity

Minimum body size at sexual maturity

The body size at which the gopher tortoise reached sexual maturity appeared to have varied more by location than by latitude (Table 9). This finding was especially evident in females, whereas in males the largest values were to be found in the northernmost sites. At least among males complicating this topic were the varying criteria used for determining sexual maturity. For example, using fertility as the criterion, ABS males reached maturity at smaller body sizes (this study; White et al., 2018) than those of south-western Georgia (Landers et al., 1980). Among males in west-central Florida, plastral concavity was detected at 200 mm CL and full concavity at 240–250 mm CL, which yielded a minimum size at sexual maturity of 240 mm CL (Mushinsky et al., 1994). In northern Florida, the smallest males measured 177 mm CL based on development of gulars and plastral concavity (Diemer & Moore, 1994). Gular projections were found to be a sexually-dimorphic trait, with those of adult males being relatively longer and more deeply-notched than those of adult females (Meshaka & Layne, 2015). In south-western Georgia, males were sexually mature at 230–240 mm CL, and all males visiting females were at least 240 mm CL (Landers et al., 1982). However, males with active sperm were as small as 203 mm CL.

Mean adult body size

Body sizes of adult gopher tortoises were found to be largest in northern and southern populations and smallest at sites of intermediate latitude, temperature, productivity, and seasonality (Ashton et al., 2007) (Table 9). Data from this study corroborated findings by Ashton et al. (2007), who also used data from the ABS.

Sexual dimorphism in body size

Across its geographic range, degree of body size dimorphism tended to be stronger at minimum body size at sexual maturity than at mean adult body size, which was generally near unity (Table 9). Still, among the 20 largest adults of each sex, males were smaller and weighed less than did females (Meshaka & Layne, 2015). Predation was found to shape body size and body size dimorphism in this species. For example, females were larger than males at a pine flatwoods/hardwood hammock site in west-central Florida (Godley, 1989). Both sexes from that site were larger than the equal-

sized males and females from a primarily pasture with some upland hammock site in west-central Florida (Godley, 1989). The smaller size and lack of sexual dimorphism at the latter site was thought to be a result of human harvest pressure on large adults (Godley, 1989). Estimated by burrow width, northern Florida tortoises (Alford, 1980) were smaller than those of Cape Sable, a coastal strand in extreme southern Florida (Kushlan & Mazzotti, 1982, 1984). The largest tortoises from Alford's (1980) study were those from ruderal settings. Difference in body size dimorphism between the two ABS sites seem best explained by differential human predation, whereby males of the roadside colony were smaller perhaps because of differential susceptibility to harvest than those of the protected west section population. Geographic variation in sexual dimorphism in this species has not been restricted to body size. Mushinsky et al. (1994) noted greater dimorphism not only in carapace length, but also in bridge width and bridge thickness in Georgia populations than in gopher tortoises in Tampa.

Body condition

In general, condition values were higher among individuals of the more productive East Section than the West Section. In contrast, females did not differ in condition between scrub and flatwoods habitats at the Avon Park Air Force Range in south-central Florida (Rothermel & Castellón, 2014). Condition of individuals from neither our study nor those of Rothermel & Castellón (2014) was affected by season. Our findings of overall lower values among males appeared to have been related to their greater movements regardless of location.

Growth and survivorship

Generally speaking, the gopher tortoise took longest to mature in northernmost latitudes (Table 9). Our findings conformed to that pattern. However, habitat quality may have played a role in the differences in age of sexual maturity between two populations from nearby sites (Godley, 1989; Mushinsky et al., 1994). For example, in southern Alabama and south-western Georgia, differences in growth and survivorship were related to land management practices in populations located not far from one another (Tuberville et al., 2014). In slash pine plantations in south-central Alabama, gopher tortoises required 20 years to mature, presumably because of human disturbance of the site that resulted in loss of forbs (Aresco & Guyer, 1999a). Notwithstanding natural or human-mediated differences in habitat quality to explain differences in growth rates among sites, geographic differences exist in growing seasons. In that regard, most growth occurs during May–September in northern Florida (Auffenberg & Iverson, 1979) as compared to April–October in south-western Georgia (Landers et al., 1982).

The asymptotic body size of the gopher tortoise on the ABS was reached at smaller sizes in males (260.7 mm PL, 281.4 mm CL) than in females (272.4 mm PL, 302.8 mm CL). The same trend was evident in south-central Alabama, where asymptotic body size was smaller in males (270.6 mm CL) than in females (322.6 mm CL)

Table 9. Summary of body size values and age at maturity for the gopher tortoise (*G. polyphemus*) across its geographic range

| Location | Minimum adult body size - Male (age at maturity) | Minimum adult body size - Female (age at maturity) | Mean adult body size - Male | Mean adult body size - Female | Mean male body size : mean female body size |
|---|--|--|--|--|---|
| SW Florida (McLaughlin 1990) | 225 mm CL (9–13 yrs) | 282 mm CL (14 yrs) | 288.4 mm PL/ 284.7 mm CL | 302.8 mm PL/ 302.9 mm CL | 0.95 |
| SE Florida (Ashton et al. 2007) | | | | 308 mm CL | |
| SE Florida Jon A. Moore, pers. comm) | | | 290 mm CL | 310 mm CL | 0.94 |
| SE Florida (Sano 2014) | >240 mm CL (7–9 yrs) | >240 mm CL (7–9 yrs) | | | |
| SC Florida This study | 186 mm PL/ 209.8 mm CL (7.4 yrs) | 225 mm PL/ 253.1 mm CL (12.7 yrs) | East = 221.6 mm PL West = 227.8 mm PL | East = 252.5 mm PL West = 246.9 mm PL | East = 0.88 West = 0.92 |
| SC Florida (White et al., 2018) | 209 mm CL | | 275.6 mm CL | | |
| SC Florida (Rothermel and Castellón 2014) | | 251 mm PL/ 254 mm CL | | 274.7 mm PL/ 278.4 mm CL | |
| WC Florida (Mushinsky et al. 1994) | 240 mm CL (probably 9–10 yrs) | 242–315 mm CL (9–10 yrs) | | | |
| WC Florida (Godley 1989) | (16–19 yrs) | 255 mm CL (16–19 yrs) | 275.1 mm CL | 299.8 mm CL | 0.92 |
| WC Florida (Godley 1989) | | | 244.4 mm CL | 247.4 mm CL | 0.99 |
| WC Florida (Small and MacDonald 2001) | | 187 mm CL (5–12 yrs) | | | |
| WC Florida (Linley 1986) | | (13 yrs) | | | |
| N Florida (Auffenberg and Iverson 1979) | 230 mm CL | 238 mm CL | | | |
| N Florida (Iverson 1980) | | 220–230 mm PL (10–15 yrs) | | | |
| N Florida (sandhill) (Diemer 1992) | | | 234.1 mm CL | 258.8 mm CL | 0.91 |
| N Florida (modified habitat) (Diemer 1992) | | | 238.9 mm CL | 266.7 mm CL | 0.90 |
| N Florida (modified habitat) (Diemer 1992) | | | 234.8 mm CL | 262.4 mm CL | 0.89 |
| N Florida (Diemer and Moore 1994) | 177 mm CL (9–13 yrs) | 232 mm CL (14–18 yrs) | | | |
| N Florida (Smith 1995) | | | 244.3 mm CL | 255.2 mm CL | 0.96 |
| SW Georgia (Landers et al. 1980) | 230–240 mm CL; active sperm at 203 mm CL | + 255 mm CL | | | |
| SW Georgia (Landers et al. 1982) | (16–18 yrs) | 250–265 mm CL (19–21 yrs) | | | |
| SC Alabama (Aresco and Guyer 1999a) | | (20 yrs) | | | |
| South Carolina (Wright 1982) | (12 yrs) | | | | |

(Aresco & Guyer, 1999a).

Survivorship in the gopher tortoise was lowest in the earliest life history stages. For example, 89 % of nests were destroyed in south-western Georgia, with a success rate of about one clutch per 9.5 nests (Landers et al., 1980). During a two-year period at a site in South Carolina 70.8 % of gopher tortoise nests were destroyed, and annually, 70 % of hatchlings were lost to predation (Wright, 1982). In southern Mississippi, 28.8 % of nests hatched (Epperson & Heise, 2003), the success rate of

which did not vary between ruderal and forested sites.

Mortality during the first year of life was over 90 % in central Florida (Witz et al., 1992), and bimonthly survival rates of hatchlings in central Florida exceeded 60 % (Wilson, 1991). Mortality during the first year of life exceeded 90 % in northern Florida (Alford, 1980), and in southern Mississippi, 65 % of hatchlings were dead within 30 days of life (Epperson & Heise, 2003).

Among adult tortoises on the ABS, residents fared better than relocated individuals. Eleven percent of

resident tortoises on the ABS were still alive after 15 years and 64 % survived at least one month, whereas all relocated tortoises were gone by 14 years and only 41 % survived for more than one month (Layne, 1989).

In this study, we found no difference in modelled annual survival of males and females. The estimates across the population agreed well with the known animals and empirical data outlined above as modelled survival would lead to a 25 % survival over 15 years. Capture probabilities differed over time, sex, and age, however, were relatively high. Given that the searches were conducted by the same biologist over the entire study, knowledge of individual habitats, tortoise patterns, and search image may have increased capture rates. In south-western Georgia, some tortoises were thought to live 80–100 years (Landers et al., 1982). Using the 12.7-year age at maturity from the East Section and a survival rate of 0.919 from this study, approximately 0.3 % of the adult population could be expected to reach 80 years of age.

Recapture Rates

On the ABS, males were captured more often than females during most of the year (this study; Douglass & Layne, 1978). To that end, south Florida males foraged and moved about between female burrows, while females generally remained closer to their own burrows (Karlin, 2002). However, the finding of greater male movements in south and south-central Florida contrasted with findings in north-central Florida, where recapture rates increased over time but were still generally low and similar between sexes (Smith, 1995). At a sandhill and two modified habitats in northern Florida, recapture rates were generally higher for adults than juveniles (Diemer, 1992a).

Activity

Seasonal

On the ABS, we found the gopher tortoise to be active throughout the year, especially during April–August, and least active during the winter. Likewise, continuous activity with reduction during the winter in this species was found on the ABS (Douglass & Layne, 1978), on Sanibel Island in south-western Florida (McLaughlin, 1990), in Jupiter, Florida, where it also breeds throughout the year (Moore et al., 2009), a coastal strand in Vero Beach, Florida (Jon A. Moore, pers. comm.), and farther north in Brevard (Hollister, 1951) and Putnam (Hubbard, 1893) counties. In north-central Florida, individuals were active continuously, especially during March–November, with most captures during May–October (Smith, 1995). However, in north-central Florida, individuals could also be active during late November–late-February (Clements, 1956). Similarly, in the northern edge of its geographic range, the species has an overwintering period (Speake & Mount, 1973). In south-western Georgia, most activity occurred during May–August, and tortoises were dormant during December–March (Landers et al., 1982). In south-western Georgia, little activity occurred during November–February and all individuals were active by 1 April when maximum air temp was at least 27°C (McRae

et al., 1981).

Diel

In agreement with others (Pope, 1939; Oliver, 1955), the species was diurnal on the ABS (this study; Douglass & Layne, 1978). However, foraging at dusk occurred during hot weather (Oliver, 1955), adults would occasionally leave their burrows at night (Alexy et al., 2003), and nocturnal forays by juveniles occurred after storms (Pike & Grosse, 2006). On the ABS, the gopher tortoise exhibited a unimodal diel pattern throughout the year, having peaked during the hottest times (1300–1600hrs) (Douglass & Layne, 1978). Our findings were similar to those of Douglass & Layne (1978), but the ranges were greater during the hottest months. We also qualify this conclusion with the possibility that analysis of diel activity that accounts for cloud cover and microhabitat could result in differences in patterns of its diel pattern of activity. Hubbard (1893) also reported unimodality in diel activity in Florida.

Individuals from a Jupiter, Florida, population exhibited a unimodal activity pattern during the colder months and switched to a bimodal activity pattern in the summer, foraging early in the morning and near dusk to avoid the hottest times of the day (Jon A. Moore, pers. obs.). In South-western Georgia, diel pattern of activity varied seasonally whereby the pattern was unimodal during May–June and September–October and was bimodal during March–April and June–August (McRae et al., 1981).

Average body temperature of active gopher tortoises was 34.7° C (Douglass & Layne, 1978). Apparently, daytime temperatures on the ABS did not exceed limits for activity, even if individuals were in the shade. However, males were active earlier and later in the day than were females, which was thought to be in response to more opportunities for mating (Douglass & Layne, 1978). In south-eastern Florida, activity was associated with mean temperatures of 31.8° C carapace surface temperature and 32.3° C cloacal temperature (Schaffner, 2015). Carapace surface temperature averaged 27.6° C among individuals basking on the apron of the burrow and 26.4° C among individuals inside the burrow tunnel (Schaffner, 2015).

Movements out of burrows in south-eastern Florida are typically associated with air temperatures ranging 21–34° C (Jon A. Moore, pers. comm.) but avoided foraging when air temperatures > 32° C (Schaffner, 2015).

Movements

All segments of the gopher tortoise population that we studied moved most often and farthest during the warmer months, and movements of adults were significantly greater than those of juveniles. Somewhat surprisingly, only the variance in distance between captures was greater in males than females, which was presumably in response to courtship activities of wandering males. Similar long movements between the sexes on the ABS may have been a response to localised sources of high-quality food on the East Section and overall lower productivity of the West Section.

Home range size-

Home range size averaged larger among males, females, and juveniles on the ABS than elsewhere. For example, in south-western Florida, home range size was significantly larger in males (1.10 ha) than in females (0.06 ha) McLaughlin, 1990. In central Florida, overall home range size averaged 1.1 ha (Doonan, 1986), with a follow-up at that site of 0.63 ha for males and 0.21 ha for females (Bard, 1989). In northern Florida, roadside strip surrounding a mature slash pine plantation, average home range size varied among males (0.88 ha), females (0.31 ha), subadults (0.05 ha), and juveniles (0.01 ha) (Diemer, 1992b). Female home range size in north-central Florida females averaged 0.48 ha in sandhill and 0.11 ha in old field (Smith 1995). Males made many courtship-related short distance movements in the spring in south-western Georgia (Landers et al., 1980). In south-western Georgia, home range size was bigger in males (0.45 ha) than in females 0.08 ha) but thought to be larger if studied for longer time (McRae et al., 1981). Home range size was thought also to have increased with a decrease in herbaceous biomass (Auffenberg & Iverson, 1979), and ultimately it was influenced by habitat quality (Diemer, 1992b). Roads were found to have the ability to elongate the home range size (McRae et al., 1981; Douglass, 1986; Diemer, 1992b). All three of the aforementioned factors affecting home range size in the gopher tortoise appeared to have influenced our findings on the ABS. First, the long-term nature of the study could capture a great deal of the variability in seasonal and annual movements. Second, although the East Section sandhill was associated with better forage than the West Section scrub, overall, the infrequently burned sandhill and the frequently burned scrub were not high-quality habitats from a food productivity standpoint. Third, the road bisecting the two sections provided a unique linear habitat that was exploited by males, females and juveniles.

Habitat use

We found that, in descending order, sandhill, flatwoods, sand pine scrub, and human-modified habitats were used most by the gopher tortoise on the ABS. Along a small mammal trapping grid on the ABS, gopher tortoises were more frequently encountered in low flatwoods-palmetto and mature sand pine scrub-oak phase, and in scrubby flatwoods- inopina oak phase, low flatwood- grass, or bayhead (Meshaka & Layne, 2015). Fire periodicity was shown to affect burrow density on the ABS (Ashton et al., 2008). A preference by this species for open sandy habitat that is naturally maintained by fire on the ABS conforms to findings across its geographic range. In south-eastern Florida, burrows were most associated with bare sand and were most numerous and regularly dispersed in wet prairie associations, which was richest in forbs among their sites (Stewart et al., 1993). This species was most abundant in grassy, open canopy habitat, higher in sandhills and low in sand pine scrub (Auffenberg & Franz, 1982) and was considered primarily a sandhill grazing species (Landers, 1980). The importance of open canopy

was such that in association with increasing canopy cover over time, burrow abandonment was recorded at a rate of 22 % each year over a five-year period in a pine plantation (Aresco & Guyer, 1999b).

Burrow dynamics

Tortoises

On the ABS, hatchlings used existing burrows or would hide in debris until the following spring, at which time they began making their own burrows (Douglass, 1978). Body size correlated very closely with burrow size on the ABS (this study; Martin & Layne, 1987), in south-western Florida (McLaughlin, 1990), and in northern Florida (Alford, 1980).

Commensal vertebrate species

Far and away, the most numerous species found in gopher tortoise burrows across treatments on the ABS was the exotic greenhouse frog, *Eleutherodactylus planirostris* (66.2 %) (Lips, 1991). Species richness and abundance varied among habitats. The numbers of species inhabiting burrows were similar among turkey oak (n = 10), sand pine scrub (n = 11), and scrubby flatwoods (n = 13) sites, with fewest numbers of species in unburned scrubby flatwoods (n = 5) as compared to burned scrubby flatwoods (n = 12) (Lips, 1991). The highest number of individual animals found in burrows was in turkey oak (n = 139), followed by sand pine scrub (n = 97) and scrubby flatwoods (n = 83) (Lips, 1991). Again, fewer individual animals occurred in unburned scrubby flatwoods (n = 37) than in burned scrubby flatwoods (n = 46) (Lips, 1991). Thus, gopher tortoise burrows are important to a wide range of species on the ABS as they are elsewhere (Jackson & Milstrey, 1989; Ashton & Ashton, 2004.)

Reproduction

Male combat

Male-male combat on the ABS was observed in January and described in detail (Hailman et al., 1991).

Courtship and mating

Courtship and mating occurred during more months in southern Florida populations than in northerly populations. Testes from gopher tortoises that we examined on the ABS during May–October indicated fertility during those months. Courtship was observed in the field in May (Layne in Douglass, 1976) and during March–November among captives (Douglass, 1976). In south-eastern Florida, courtship and mounting was observed year-round, the peak incidence of which occurred during August–December (Moore et al., 2009). On Sanibel Island in south-western Florida, mating was observed 12 times during 18 May–27 June, three times during 3 July–23 August and once in September (McLaughlin, 1990). In north-eastern Florida, reproductive activity occurred during April–November and males head-bobbed at the burrow mouths of females most commonly in September (Butler & Hull, 1996). Courtship occurred during September–October in northern Florida (Diemer, 1992b) and during spring–fall in south-western Georgia (Landers et al., 1980). In

south-western Georgia, mating appeared to be restricted to late April–early-June when chin glands were active in females (Landers et al., 1980).

Nesting

Southern Florida populations of the gopher tortoise nested over a longer period than those of northerly sites. On the ABS, two dissected females containing shelled eggs were found in May, a nest was found in May, and two additional nests were found in June (Meshaka & Layne, 2015). On the ABS, nesting was observed during May–June (Douglass, 1976), and the 32 mm follicle we found in an April female on the ABS fit within the ovarian cycle of northern Florida gopher tortoises (Iverson, 1980). On 11 May in Florida, a female was observed digging a nest on a mound of old peat mulch. In south-western Florida, shelled eggs were also present in females during April–May; however, inferentially nesting may have been possible from Fall through Spring as well (McLaughlin, 1990), partially-shelled oviductal eggs and ovulated follicles were found in a female from Palm Beach County on 3 April (Iverson, 1980), and shelled eggs were detected in females as early as 7 April in Jupiter, Florida (Moore et al., 2009).

North of the ABS, studies showed that the nesting season began later and ended by early- to mid-summer; no nesting was observed during 5 March–19 April in northern Florida (Hallinan, 1923); field records showed nesting during May–June in northern Florida (Iverson, 1980); nesting records during late May–early June (Hallinan, 1923; Iverson, 1980); nesting in early-June (Diemer & Moore, 1994) and the month of June (Smith, 1995) in northern Florida; nesting during mid-May–late-June in south-western Georgia (Landers et al., 1980); nesting during mid-May–mid-July with peak nesting during late-May–early-June in southern Mississippi (Epperson & Heise, 2003); and nesting during late-May–June in South Carolina (Wright, 1982).

Clutch size—Clutch sizes of the gopher tortoise were thought to be larger in southern Florida than in northern Florida (Iverson, 1980). Subsequently, clutch size was found to be largest in the southern portion of its geographic range in association with greater productivity of resources and less seasonality (Ashton et al., 2007). This is particularly evident along the southern coast. To that end, mean clutch size was 6.9 eggs in south-western Florida (McLaughlin, 1990), 6.3 eggs on the ABS (Ashton et al., 2007), 5.8 eggs at the Avon Park Air Force Range (Rothermel & Castellón, 2014), 8.2 eggs at Okeechobee County Park in Palm Beach County (Ashton et al., 2007), 7.46 eggs on Merritt island (Demuth, 2001), and 10.1 eggs (range = 8–13) from Jupiter, Florida, where the largest clutch radiographed was 13 eggs, although a nest with 18 eggs was later found (Moore et al., 2009).

Northward, mean clutch size of the gopher tortoise was shown to have decreased in size. In central Florida, mean clutch size was 7.6 eggs (Godley, 1989). In west-central Florida, the mean clutch size of 8.5 eggs did not differ significantly between resident and trans-located tortoises but increased with an increase in female

body size (Small & MacDonald, 2001). In west-central Florida, the mean clutch size was 7.8 eggs (Linley, 1986). In Northern Florida, mean clutch size based on a combination of estimates (all similar) was 5.2 (Iverson, 1980). In northern Florida, mean clutch size was 5.8 eggs (Diemer & Moore, 1994). In north-central Florida, mean clutch size was 5.76 eggs (Smith, 1995). In north-eastern Florida, mean clutch size was 5.04 (Butler & Hull, 1996). In southern Mississippi, mean clutch size was 4.8 eggs (Epperson & Heise, 2003). In south-western Georgia, clutch size averaged 7.0 eggs (Landers et al., 1980). Average clutch size in north Florida (mean = 5.3; range = 1–9) and egg dimensions were smaller than those of south-western Georgia and thought by Landers et al. (1980) to be explained by the smaller body size of those animals. In South Carolina, mean clutch size was 3.8 eggs (Wright, 1982). Body size and clutch size were not positively related on the ABS (Ashton et al., 2007) but were so at Okeechobee County Park (Ashton et al., 2007), at Avon Park Air Force Range (Rothermel & Castellón, 2014), in northern Florida (Iverson, 1980; Diemer & Moore, 1994; Smith, 1995), and in south-western Georgia (Landers et al., 1980). At Okeehelie County Park, the largest clutch sizes were produced by intermediate-sized females, indicating age-related reproductive senescence (Ashton et al., 2007).

Annual clutch production

No evidence of multiple clutch production was found in a single dissected female from ABS, which was in keeping with findings of single annual clutch production in this species from northern Florida (Iverson, 1980; Taylor, 1982; Diemer & Moore, 1994; Smith, 1995), south-western Georgia (Landers et al., 1980), and South Carolina (Wright, 1982).

Incubation times

Incubation times were not measured on the ABS; however, in general eggs of the gopher tortoise took approximately three months to hatch: 101 days in the aforementioned peat mound (Meshaka & Layne, 2015), an average of 88.5 days (88 and 89 days) in south-western Florida (McLaughlin, 1990); a 91-day incubation time for a clutch laid in April 2013 in south-eastern (Vero Beach) Florida (Jon A. Moore, pers. comm.); a range of 91–105 days (Linley & Mushinsky, 1994) and 56–102 days (Small & MacDonald, 2001) in west-central Florida; an average of 87.4 days in north-central Florida (Smith, 1995); an average of 105 days in north-eastern Florida (Butler & Hull, 1996); a probable range 80–90 days in northern Florida (Iverson, 1980); and an average of 102 days in south-western Georgia (Landers et al., 1980). Incubation time averaged 88 days in southern Mississippi in both forested and ruderal sites (Epperson & Heise, 2003). If a geographic trend in incubation time exists for the gopher tortoise, it was not apparent from these aforementioned studies.

Hatching season

On the ABS, hatchlings appeared during late August–early October (Douglass, 1978). In this study, the smallest

individuals appeared during April–September, the earliest of which were presumed to have hatched late the previous fall. In south-western Florida, the hatching season was during late-May–late-September, suggestive of extended reproduction, with most hatching ending by late August (McLaughlin, 1990). Farther north, hatching was restricted to late summer and fall as reported in the following studies; at least mid-August–September in northern Florida (Iverson, 1980); late-August–early-October in north-eastern Florida (Butler & Hull, 1996); and the month of September (Arata, 1958) and during late-August–early-October in north-central Florida (Smith, 1995).

Hatchling size

Mean hatchling size on the ABS was 47.7 mm PL (Douglass, 1978) and smallest hatchlings measured 42 mm PL (this study) and 43 mm PL (Douglass, 1978). The means of the following data were recorded from four hatchlings from a single clutch from the ABS: 31.1g, 46.6 mm CL, 41.8 mm CW, 43.0 mm PL, 36.5 mm PW, and 5.1 mm anterior projection of the gular scute (Meshaka & Layne, 2015). In west-central Florida body size of hatchlings ranged 45.9–47.7 mm PL (Small & MacDonald, 2001), and in north-central Florida, mean hatchling size differed between two years (48.0 vs. 42.3 mm CL) (Smith, 1995).

Diet

On the ABS, the gopher tortoise ate a wide range of vegetation but foraged primarily on grasses (this study; Meshaka & Layne, 2015). An interest in grasses and nutritious forbs was evident in adults and juveniles in many parts of its geographic range. Pusleys and bromeliads were also found in many stomachs. In west-central Florida (MacDonald & Mushinsky, 1988) and south-western Georgia (Garner & Landers, 1981), individuals also ate a lot of grasses but preferred the more nutrient-rich forbs. In South Carolina, wiregrass also comprised much of its diet (Wright, 1982). Among adults, *Aristida* was important in many places (Auffenberg, 1969; Fletcher, 1899; Wright, 1982), perhaps in relation to its abundance. In a west-central Florida sandhill, adults ate a wide range of plant taxa (MacDonald & Mushinsky, 1988). Scat analysis revealed *Aristida* and Poaceae at the genus and family level, respectively, to be the most abundant food items in a west-central Florida population (MacDonald & Mushinsky, 1988). Also commonly eaten at MacDonald & Mushinsky's (1988) site were *Pinus*, *Quercus*, *Galactia*, *Cnidocolus*, *Tillandsia*, *Pityopsis*, and *Richardia*. Differentially chosen plants were *Galactia* and *Tephrosia*, *Cnidocolus*, *Pinus*, *Quercicus*, *Vaccinium*, *Richardia*, and *Rubiaceae*, thereby eating *Aristida* at its occurrence (MacDonald & Mushinsky 1988). More so than smaller individuals, adults ate plants with some form of chemical protection (MacDonald & Mushinsky, 1988). Likewise, in south-western Georgia Garner and Landers (1981) noted that *Aristida* was poorly exploited in areas where more nutritious forbs were available. Ingestion of calcium-rich stones, fossil shells, and small mammal bones was reported in females whose developing eggs were undergoing calcium deposition (Moore & Dornburg,

2014). The importance of calcium in the diet could explain the rocks and squirrel bones found in the ABS individuals. In southern Florida, scavenging for animal protein in carcasses was also reported for the gopher tortoise (Meshaka & Layne, 2015) which may have been associated in part with a search for bones as is known to be the case with ingestion of mammalian scats (Moore & Dornburg, 2014).

Juvenile gopher tortoises in a west-central Florida sandhill generally ate forbs when seasonally abundant then switched to grasses, often to the level of its availability (Mushinsky et al., 2003). Overall, juveniles elected on average less than ½ the genera that they would encounter on a given foraging bout and avoided all together *Aristida*, a very common grass (Mushinsky et al., 2003). Coprophagy was observed by members of the ABS population. Among juveniles, coprophagy was found to inoculate their gut with microbial symbionts helpful in digestion of plants (Bjorndal, 1987; Moore & Dornburg, 2014). To that end, the cellulolytic microflora that degrades cellulose and hemicellulose resulted in a high (73 %) digestibility of cell walls in the diet of the gopher tortoise (Bjorndal, 1987)

Seed dispersal- On the ABS, gopher tortoise-dispersed seeds of the exotic Bahia Grass (*Paspalum notatum*) germinated at lower frequencies than those of its native congener, *P. setaceum* (Carlson et al., 2003). Both of these grasses were most common in gopher tortoise scats on the ABS but were accompanied by a wide range of other plants, especially *Pinus elliotti*, *Galactia* sp., *Vaccinium myrsinites*, *Quercus geminata*, *Gaylussacia dumosa*, and roots (Carlson et al., 2003). The relative frequency of seed species found in scats was highest for *P. notatum*, *P. setaceum*, and *Diodia teres* (Carlson et al., 2003).

Predators

Eggs

On the ABS, nest predators were the eastern indigo snake (Layne & Steiner, 1996), raccoon (*Procyon lotor*), gray fox (*Urocyon cinereoargenteus*), and especially the nine-banded armadillo (*Dasypus novemcinctus*) (Douglass & Winegarner, 1977; Meshaka & Layne, 2015). Also on the ABS, an egg fragment and other food remains were found beneath the nest of a Cooper's hawk (*Accipiter cooperii*) (Meshaka & Layne, 2015). The raccoon was an important nest predator as reported by Hallinan (1923) and individuals depredated nests in north-central Florida (Smith, 1995). Nest predators in south-western Georgia were the raccoon, gray fox, striped skunk (*Mephitis mephitis*), and opossum (*Didelphis marsupialis*) (Landers et al., 1980).

Hatchlings

On the ABS, recently hatched tortoises were found in the diet of the eastern indigo snake (Layne & Steiner, 1996) and the remains of a fall hatchling were recovered from the stomach of an eastern coachwhip the following May (Douglass & Winegarner, 1977). The red-tailed hawk (*Buteo jamaicensis*) was reported as a predator of juvenile gopher tortoises on the ABS (Fitzpatrick & Woolfenden, 1978). In St. Petersburg, Florida, remains

of a juvenile were removed from a wild-caught savannah monitor (*Varanus exanthematicus*) (Owens et al., 2005). Geographically expanding populations of the Argentine giant tegu (*Salvator merianae*) and the Nile monitor (*Varanus niloticus*) (Meshaka, 2013) provide novel threats to eggs or young of the gopher tortoise. In Jupiter, Florida, remains of a juvenile were recovered from coyote (*Canis latrans*) scat (Moore et al., 2006). In north-central Florida, hatchlings were killed by ants (*Solenopsis geminata*, *S. invicta*, *S. pergandei*, and *Conomyrma bossuta*) (Smith, 1995). In south-western Georgia, fire ants were predators of hatchlings (Landers et al., 1980). In southern Mississippi, hatchlings were killed primarily by mammals and secondly by fire ants (*S. invicta*) (Epperson & Heise, 2003). In Alabama, a hatchling was removed from the stomach of an Eastern Indigo Snake (Mount, 1975).

Adults

On the ABS, the gopher tortoise was subject to poaching at varying intensities (Meshaka & Layne, 2015). Sub-adults were attacked by dogs (Douglass & Winegarner, 1977). Dogs were considered to be responsible for the scarcity of gopher tortoises at one of the more developed sites in a south-western Florida study (McLaughlin, 1990). In west-central Florida, three juvenile gopher tortoises were lost presumably to raptors (Wilson, 1991). Adults were eaten by canids (Causey & Cude, 1978) and Black Bears (Landers et al., 1982).

Unnatural sources of mortality

Railroad-related mortality was observed on the ABS, whereby animals were trapped between the rails and presumably died of heat exposure and by vehicles (Meshaka & Layne, 2015). Railroad mortality was also observed in St. Lucie, which involved a 230 mm CL male (Engeman et al., 2007).

Summary

McCoy & Mushinsky (1992) found that protection of even large parcels of land was in and of itself no guarantee of population health of the gopher tortoise, and their difficulty in making temporal comparisons of abundance based on earlier surveys by others underscored the need for more systematic approaches to population census. Long-term study sites such as the ABS, with multiple habitat types and a diverse fire management schedule have provided the conditions necessary to examine the natural and human-mediated responses of the gopher tortoise to spatial differences in habitat type and quality as well as to temporal responses at a scale that is meaningful to this long-lived species. To that end, the growth to maturity, reproductive aspects, and seasonal activity were found to fit within geographic trends. We suggest that habitat quality affected population size, body condition, and home range size of this large, grassland species. Although extensive human predation appeared to best explain differences in the population structure on the ABS, body size was found to conform to predictions based on a combination of geography, climate, seasonality, and productivity.

Two human-mediated threats to the integrity of the biotic community of the ABS are likely threats to its gopher tortoise population - isolation and the exotic Argentine giant tegu. The ABS is an island of an archipelago of protected lands on the Lake Wales Ridge. Ever more separated from other islands by habitat destruction, populations of many of its species risk increasing isolation from other populations. For the gopher tortoise, this threat alone warrants dedicated continuation of monitoring of the ABS population. The Argentine giant tegu is an established member of the exotic herpetofauna of Florida (Meshaka, 2013). It occurs in two disjunct regions of Florida: Hillsborough and Polk counties and in Miami-Dade County. In the former range, this lizard occurs in xeric uplands of the Balm-Boyette Scrub Preserve and the Mosaic phosphate lands, where individuals use gopher tortoise burrows (Enge, 2007). Males can grow to an excess of 1.22 m in total length. The species is omnivorous and a documented predator of crocodylian and chelonian eggs in southern Florida (Mazzotti et al., 2015). The Argentine giant tegu represents a threat to the gopher tortoise as a predator of its eggs and hatchlings and, more broadly, represents a threat to other sandy upland species that it can capture and eat. The Argentine giant tegu will inevitably disperse onto the Lake Wales Ridge and will have a high likelihood of colonisation success on the ABS. Establishment of this species on the ABS will bring with it a high likelihood of significant alteration of the community structure and population sizes of many of the station's vertebrate species. Gopher tortoise surveys should include monitoring burrow entrances in part by periodically sweeping burrow entrances to detect the presence of resident tegus.

Our findings examined responses of the gopher tortoise to natural processes and human-mediated perturbations that were uniquely combined on the ABS. Continued study of this population can reveal the kind and extent of responses to various land management treatments, effects of isolation, and the inevitable colonization of a predatory exotic species in a well-documented site.

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Hotspot of tadpole abnormality in suburban south-west Florida

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A high concentration of Cuban treefrog (*Osteopilus septentrionalis*) tadpoles displaying morphological abnormalities was discovered in an untreated swimming pool in Bonita Springs, Florida. This find initiated a 4-year survey (2012-2015) of surrounding roadside drainage ditches that had been treated with insecticide for mosquito control. The study was extended to the populations of Ave Maria, Florida, and Everglades National Park. The core data set of 36,550 tadpoles from the swimming pool and ditches contained 25,136 abnormal tadpoles, an abnormality average of 68.8 %, well above the 5 % minimum definition for a hotspot. The frogs from Ave Maria and the Everglades National park were 0 % abnormal. The type of tadpole abnormality differed between the suburban treated roadside drainage ditches versus the untreated swimming pool, although the same abnormalities were found in both the suburban treated and untreated water. In the untreated swimming pool, 70.1 % of tadpoles displayed abnormalities such as bent tails, abnormal limbs, and disfigured or absent mouthparts. Larvae in the untreated swimming pool metamorphosed en masse despite abnormalities. The high frequency of abnormal metamorph abnormalities found were: kyphosis, scoliosis, microcephaly, and forelimb abnormalities. In the treated roadside drainage ditches, Gosner stage 16-25 tadpoles could not undergo metamorphosis and experienced mass mortality. The abnormalities found at Gosner stage 16 of the embryo were in the head and body. Tadpoles at Gosner stages 19-25 failed to develop gills, were bloated, had growth retardation, and mouthpart abnormalities. The older Gosner stage 25-41 tadpole populations in the ditches showed bloating, lumps, emaciation, and growth retardation. A brief synopsis of *O. septentrionalis* treefrog biology is also given, including breeding congregations, average 8 hour time to hatching, and 19 days metamorphosis.

Keywords: anurans, *Osteopilus septentrionalis*, tadpoles, normal development, abnormality

INTRODUCTION

Amphibians are globally threatened, with 32.5 % categorised as endangered and 43.3 % species declining (Stuart et al., 2004, 2008). The global regions with high concentrations of decreasing amphibians have been identified as Mexico, Central America, the Northern Andes, Brazil's Atlantic forest, western Africa and Madagascar, India, south-east Asia, Indonesia and the Philippines (Ceballos et al., 2017).

In the USA, many common anuran species have been disappearing (Fisher & Shaffer, 1996; Fisher et al., 2012; Ceballos et al., 2017). A National United States Fish and Wildlife Service (USFWS) frog survey covering field seasons in 2000-2009 reported that all Florida anuran species were healthy and at a normal, low abnormality rate of 2.0 % (Reeves et al., 2013). USFWS reported that there were only three abnormality hotspots nationwide, located in the Mississippi River Valley, California, and Alaska. The three hotspots were said to be a local problem (Reeves et al., 2013). Since then, in 2018, the same lab

produced another report with survey data from 2012-2016 showing that the three nationwide abnormality hotspots had changed to 96 hotspots in the USA, located in the north-east, south-east, and western regions of the USA. Furthermore, the newly identified hotspots had 5 %-25 % abnormalities and were associated with the presence of oil and gas wells at sites, as well as a history of pesticide application-parasite infection (Haas et al., 2018).

Since 1995 when malformed frogs were discovered in south central Minnesota, USA, it has been worldwide practice to mainly survey specimens at the end of metamorphosis (USFWS, 2018; Reeves et al., 2013), or for one particular abnormality (Sanchez-Domene et al., 2018). Only recently, sampling throughout the whole metamorphic period coincided with an increase in reported frequency of hotspots in the USA (Haas et al., 2018). However, data is lacking about malformations across the whole larval period or when mass death occur, which will allow us to assess if there is a larval stage that is more susceptible to chemical effects.

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The Cuban treefrog *Osteopilus septentrionalis* (Dumeril & Bibron, 1841) is a large hylid treefrog from West Indies. In Florida it is considered to be a very common, invasive, robust species that consumes smaller native frogs (Meshaka, 2001). The IUCN considers *O. septentrionalis* a species of least concern. The frog was first reported in 1931 from Key West, Monroe County, Florida (Barbour, 1931) and has rapidly colonised most of Florida (Meshaka, 2001, 2011). *Osteopilus septentrionalis* populations in Florida were most recently surveyed in 2013 and reported to be healthy (Johnson, 2013). However, after finding malformed tadpoles of *O. septentrionalis* during a survey in suburban South-western Florida in 2012, we decided to carry out extensive population monitoring (2012-2015) in order to determine the extent of these abnormalities. Abnormal tadpoles occurred in a heavy insecticidal spray zone, which does include the spraying of a petroleum distillate spreader to control mosquitoes.

To recognise abnormal patterns during larval development in a wild population, biologists need to establish a baseline that gives clear descriptions of normal morphology and normal development timeframes. To do this, several developmental staging tables have been proposed, with that of Nieuwkoop & Faber (1994) for *Xenopus laevis* and that of Gosner (1960) for the Gulf Coast toad *Incillus valliceps* as the most commonly used. Unfortunately, a survey of staging tables among anurans has indicated that the volume of information published represents less than 1.5 % of the diversity of current amphibians, with several families in which most aspects of their ontogenies are unknown (Fabrezi et al., 2017). Therefore, we first characterised the normal development of *O. septentrionalis* and then subsequently described numerous developmental malformations and associated abnormality prevalences for multiple populations of *O. septentrionalis* in Florida, USA. Finally, we discuss the use of *O. septentrionalis* as a sentinel species to detect negative anthropogenic effects on wild fauna in Florida.

METHODS

All tadpoles examined in this survey were collected from the field between 2012 and 2015. All the animals were assessed for abnormalities on site at the time of sampling. The only rearing performed was of the collected abnormal GS 42 stage tadpoles from the untreated H artificial pond site to visualise metamorph and adult frog abnormality. There were 36,550 tadpoles examined in 240 collections from 11 sites located in suburban Bonita Springs, Florida, with additional unsprayed zone sites added in 2013 to the survey from Golden Gate Naples and the Everglades National Park (see Supplementary Materials Fig. S1). The frequency of abnormality was calculated by the total of abnormal frogs collected divided by the total number of frogs collected.

There were three types of sites in the suburban area of South-western Florida: The insecticide treated roadside drainage ditch (see Supplementary Materials Fig. S2 A- E), the untreated artificial pond (see Supplementary

Materials Fig. S2 F), and containers (see Supplementary Materials Fig. 2 G, J). The ephemeral roadside drainage ditch sites lining the streets averaged 1.83 m x 1.22 m in size with 254 mm maximum rainwater depth at mid-pool. They were treated continually with insecticide. The artificial pond was untreated with insecticide and comprised a permanent body of water filled with rainwater. The dimensions of this pond were 3.048 m x 4.572 m with a depth of 1.22 m. The “H yard” container sites (see Supplementary Materials Fig S2. G, J) were outdoor empty plastic containers naturally-filled with rainwater, and then left undisturbed. These containers held a maximum of four litres. The control site types, U.S. Wildlife Refuges and Golden Gate Naples rural, were outside the mosquito control insecticidal zones (see Supplementary Materials Fig S2. H, I, K, L) and ephemeral puddles of rainwater were similar in both size and site longevity to suburban sites. Exceptionally, the ENP site located inside the park was permanent (see Supplementary Materials Fig. S2L).

The abnormality assessment and population tracking commenced when the completely dry field sites became filled with rainwater, and continued for as long as the pools remained full of rainwater. A field site sampling visit entailed the capturing and counting of aquatic larvae from generally 2-3 net sweeps at each site location. All specimens were counted, assessed then photographed on site in the collection pan. Observations were recorded with a P510 Nikon digital camera with automatic date, time and GPS. The field data was organised by site and collection date. All animals were euthanised following international ethical procedures. All Everglades National Park frogs (Study # EVER-00493) including 1432 individuals were returned to the park and archived at the South Florida Collections Management Center.

RESULTS

The field oviposition site for *Osteopilus septentrionalis* were ephemeral roadside drainage ditches located in a suburban neighbourhood that during the hurricane season (April-November) had filled with rainwater. The ditches were all temporary bodies of water lasting from between one day to a maximum of 4 weeks (before drying up). In many cases, the roadside drainage ditch was an unstable habitat simply because it did not provide the amphibian enough time to metamorphose.

Each year between 4AM and 7AM during periods of heavy rainfall, more than 100 *O. septentrionalis* congregated for breeding for three days. Both males and females turned bright yellow during this period. The amplexant pair travelled the length of the pool, dropping partial clutches.

Time Frame for Normal Development of Larval Stages to Metamorphosis (Fig. 1 A, Table 1).

The tracking of an entire in-situ population from eggs being deposited to mass metamorphosis was observed at the untreated H artificial pond site. The time taken for a normal population of *O. septentrionalis* to develop

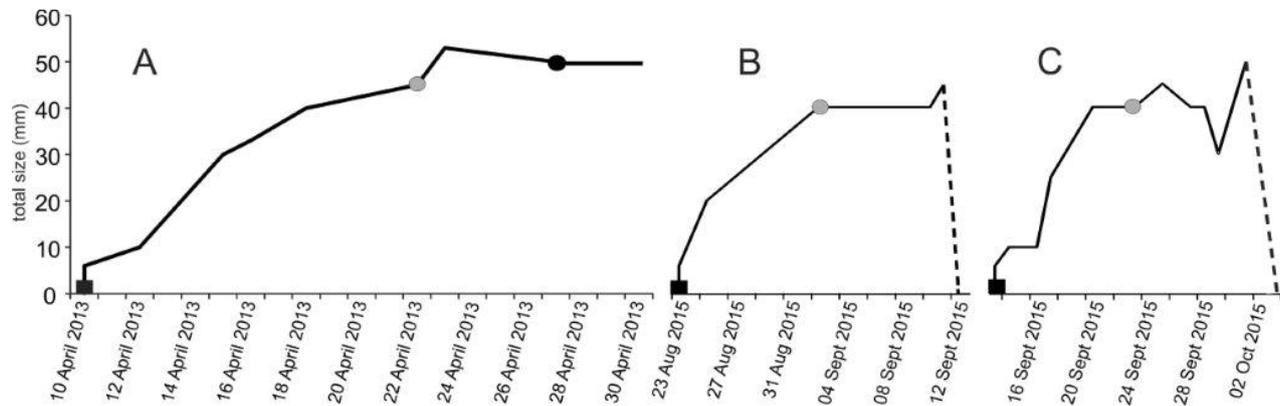


Figure 1. Survivorship Curves of three field populations in treated vs untreated water (see Table 1). (A) H artificial pond, untreated pool water. H artificial pond had normal growth with a maximum TL size of 53mm with 12th day hind limb eruption (grey circle), 17th day forelimb protrusion (black circle) and a 19-day mass metamorphosis. (B) RP1, treated pool water. (C) RP2, treated pool water. RP1, RP2 field populations had abnormal growth with a maximum size of 45mm for RP1 and 50mm for RP2. On the 15th day RP2 and on the 19th day RP1 had mass death (dotted line) of the population following hind limb eruption on the 10th day for RP2 and 12th day for RP1. Black square indicates eggs deposition.

Table 1. Aquatic tadpole length in mm (TL) and stage (eggs to metamorphosis) in days for three field site populations in treated RP1,RP2 (right) vs untreated water H artificial pond (left).

| H artificial pond date | H artificial pond size (TL) Stage | RP1 ditch pool date | RP1 Size (TL) Stage | RP2 ditch pool date | RP2 Size (TL) Stage |
|------------------------|-----------------------------------|---------------------|---------------------|---------------------|---------------------|
| 10 April 2013 | eggs | 23 August 2015 | eggs | 12 September 2015 | eggs 6 mm |
| 10 April 2013 | 6 mm 8hr hatch | 23 August 2015 | 6mm 8hr hatch | 13 September 2015 | new eggs+6 mm hatch |
| 12 April 2013 | 10 mm | 25 August 2015 | | 14 September 2015 | 10 mm |
| 15 April 2013 | 30 mm | 2 September 2015 | | 15 September 2015 | |
| 16 April 2013 | 33 mm | 5 September 2015 | | 16 September 2015 | 10 mm |
| 18 April 2013 | 40 mm | 10 September 2015 | 40mm dead | 17 September 2015 | |
| 22 April 2013 | 45 mm hind limbs | 11 September 2015 | 45mm dead | 18 September 2015 | 30 mm |
| 23 April 2013 | 50-53mm 1met | 12 September 2015 | 100 % dead | 20 September 2015 | 40 mm |
| 27 April 2013 | 50 mm forearms | | | 23 September 2015 | hind limb mass dth |
| 28 April 2013 | 1met | | | 25 September 2015 | 45 mm |
| 29 April 2013 | mass met | | | 27 September 2015 | 45 mm |
| 30 April 2013 | mass met | | | 28 September 2015 | 40 mm |
| 01 May 2013 | mass metamorphosis | | | 29 September 2015 | 30 mm dead |
| | | | | 1 October 2015 | 50 mm dead |
| | | | | 3 October 2015 | 100 % dead |

from egg to metamorph in clean water was 19 days. The hind limbs appeared in 12 days, forearms protruded in 17 days, with mass metamorphosis in 19 days. Normal development is extensively described in Supplementary Materials. The GS 42 tadpoles with a total length of 45 mm rapidly went into tail atrophy to develop into a SVL 16-18 mm GS 46 metamorph of brown, cream or olive green coloration, with or without a white lateral line. The whole series metamorphosed in synchrony and were of almost identical size (+/- 2mm).

Tadpole Malformations

Malformations are named and described by the developmental stages during which they occurred. Most developmental malformations were lethal to the entire clutch/population, but had survivors. The abnormal field populations produced abnormal clutches whose tadpoles developed in synchrony, but died upon

reaching a certain developmental stage.

Time frame for Abnormal Development of Larval Stages to Metamorphosis (Fig. 1B, C Table 1).

By the 10th to 12th day, in those field populations subject to insecticidal spray, the abnormal tadpoles were either dead, or had been eaten. When the hind limbs appeared, a mass die off began, and the field populations never achieved GS 42. By the 19th day, when the population should be mass metamorphosing, instead mass mortality was observed (Fig. 1B, C).

Stage 16 malformation (Fig. 2): there were numerous malformations that occurred between 10 and 13 hrs. The GS 16 embryo prematurely formed a GS 18 tail bud, and the head formed head tubercles where the lateral eyes should normally have been located (Fig. 2C). Other GS 16 abnormality types were observed with premature distinction of the head and body regions, without any

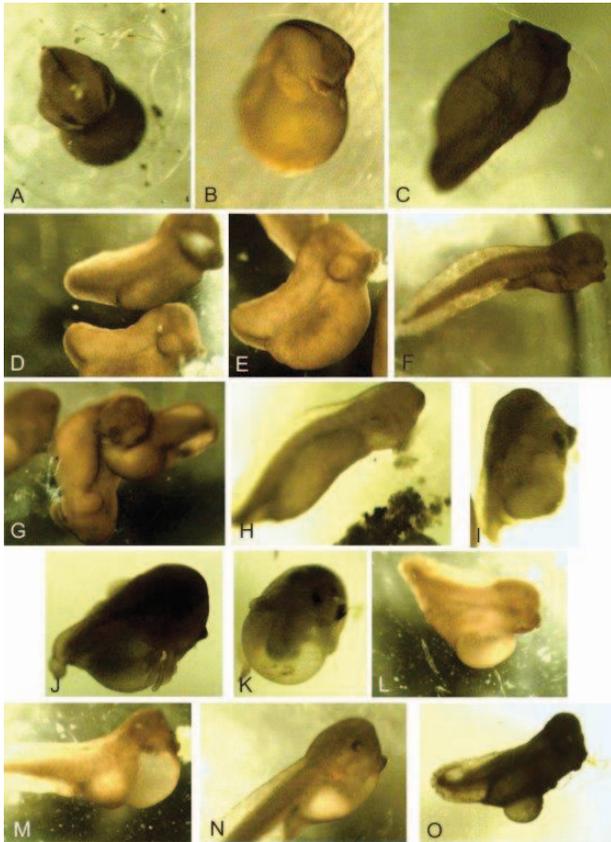


Figure 2. GS 16-GS 23 abnormal morphology roadside drainage ditch and H container. (A) GS 16 dorsal view of the enlarged head, gill buds flared, misshapen olfactory pits. (B) GS 16 dorsal view of the mouth precursor cleft. (C) GS 16 dorsal view of the tail bud, and head tubercles. (D) GS 19 lateral view of gill bud, tail fail to develop. (E) GS 19 lateral view of the gill bud, tail fail to develop. (F) GS 19 lateral view. (G) GS 19 lateral view of the tail. Body fails to lengthen, irregular shape. (H) GS 19 lateral view of the mouth with tubercles. (I)-(K) GS 19 same animal lateral view (I) 10 hrs, (J) 23 hrs. (K) 72 hrs progression of bloat. (L) GS 19 bloat lateral view. (M) GS 19 bloat lateral view. (N) GS 19 lateral view. (O) GS 19 bloat lump lateral view.

evidence of formation into a correct body or neural tube elongation. The body appeared rounded and the head region appeared abnormally enlarged and elongated. One irregular type had an asymmetrically enlarged head angled to the right (Fig. 2B). Another abnormal GS 16 type had an abnormally large elongated head with oversized, flared gill buds (Fig. 2A, B), abnormally large misshapen olfactory pits (Fig. 2A, B) with the olfactory pits incorrectly located at the stomadeum (Fig. 2B). A deep cleft formed the precursor of the mouth (Fig. 2B). The hypophyseal cleft was enlarged, and of an abnormal shape (Fig. 2A).

Stage 19 malformation (Fig. 2): malformation occurred immediately after hatching. At 24 hrs, the entire clutch typically lay motionless at the bottom, in dead carcass piles (Fig. 2D). The gill buds and tail fail to develop (Fig. 2D, E). In another GS 19 mass die off, the gill buds also failed to develop, however the tail elongates normally, with a clear dorsal and ventral tail fin (Fig. 2F). In

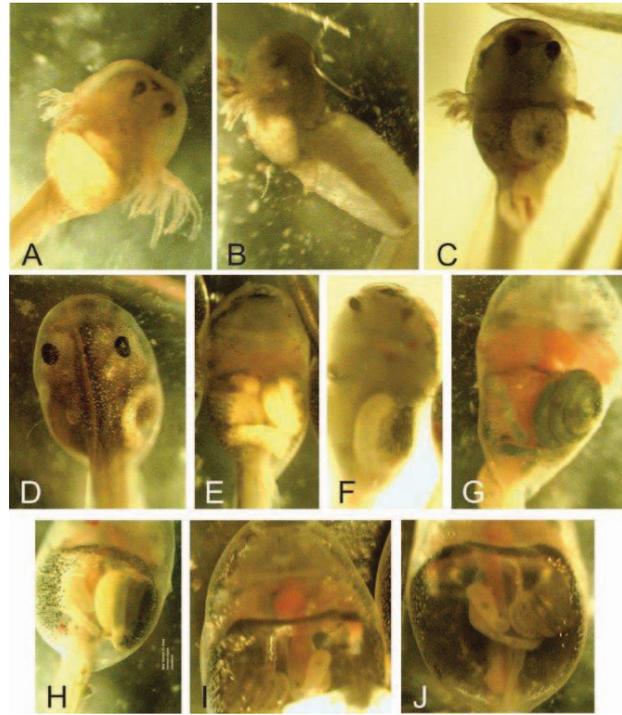


Figure 3. GS 23-25 abnormal morphology roadside drainage ditch and H container. (A) GS 23, ventral view, asymmetrical gills. (B) GS 23, lateral view, body not lengthen. (C) GS 23 intestinal location abnormality. (D) GS 25 dorsal view, intestinal location abnormality and GS 25 intestinal location abnormality. (E) GS 25, ventral view, intestinal location abnormality. (F) GS 25 intestinal location abnormality. (G) GS 25 intestinal location abnormality. (H) GS 25 organ abnormality. (I) GS 25 organ abnormality. (J) GS 25 organ abnormality.

some cases, either the tail, the body (or both structures) failed to lengthen, and were irregularly shaped (Fig. 2G). In some specimens, the mouth appeared abnormal with tubercles (Fig. 2F, H). Bloat in the body cavity was found at 23 hrs, and rapidly worsened with age to cause mortality by 72 hrs (Fig. 2I-K). Most malformations occurred in combinations with other malformations (Fig. 2H, I, M, N, O).

Stage 23 malformation (Fig. 3): the incidence of the GS 23 malformation was rare. The hatch most often could successfully internalise the gills. If the GS 23 could not internalise the gills, it was unable to lengthen its body, had asymmetrical gills, or it showed signs of intestinal abnormality (Fig. 3A-C).

Stage 25 malformation (Fig. 3-4): intestinal abnormalities included incomplete formation, malrotation, or either oversized or growth retarded organ. The location of the small and large intestine became abnormal (Fig. 3D-G). The abnormal growth of the intestine displaced the location of the other organs which were also abnormally formed in size and shapes (Fig. 3H-J). All intestinal irregularities appeared to be lethal, as all field tadpoles with intestinal malformation had a rapid, synchronous death. Another malformation found at the GS 25 was a normal intestine accompanied by a body size total length (TL) retardation (Fig. 4C) of 6 mm, compared with a normal total size of 15-20 mm.

The majority of the clutch ceased growth and remained

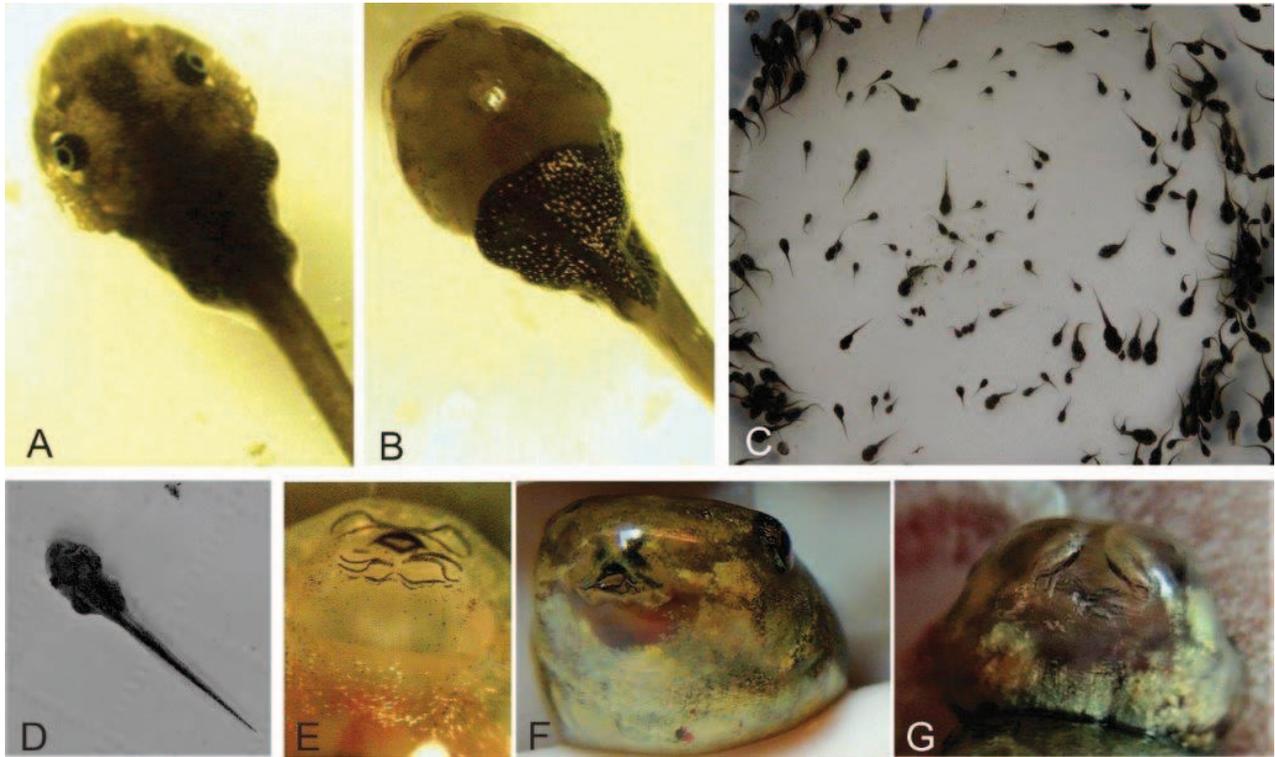


Figure 4. GS 25 abnormality roadside drainage ditch and H container. (A) GS 25, dorsal view, growth retardation, emaciation, lumps. (B) GS 25, ventral view, growth retardation, emaciation, lumps, epidermal blackening. (C) GS 25 one clutch all GS 25 growth retarded and normal growth. (D) GS 25 normal growth, emaciated, lumps. (E) GS 25 disfigured upper and lower labial tooth rows. (F) GS 25 disfigured lower labial tooth rows. (G) GS 25 cleft mouth.

at a 6 mm body size. Clutches with this severe TL body size growth retardation were observed alive, swimming, feeding and fully alert, while excreting waste through the vent tube, which had become blackened and dry (Fig. 4A, B). The field growth retarded clutches each day became more and more emaciated, and often developed lumps (Fig. 4A). The normal growers also developed lumps and became emaciated (Fig. 4D). The entire clutch soon underwent mass die off in synchrony between 2-4 weeks. The time taken for death from the emaciation and wasting away varied depending on the clutch. In some cases, the growth retardation malformation was not combined with the black shrivel epidermal blackening. There were always a few individuals in a clutch that grew to normal size. The growth-retarded 6 mm tadpoles were a valuable food source for the larger normal 15 mm clutch mates, which cannibalised their smaller siblings. The GS 25 mouthparts found more often were abnormal (Fig. 4B, E-G). The lower and upper labial tooth rows found disfigured (Fig. 4E, F), missing (Fig. 4B, F), or all missing. A rare cleft mouthpart was found (Fig. 4G).

Stage 41 malformation (Fig. 5): bloat, a common malformation, frequently occurred at the stage when tadpoles were at least 40 mm, and had fully formed hind limbs. There were two types of pronounced bloat: 1) Tadpoles turned black with the trunk and head bloated, and often lopsided (Fig 5A). The necropsied larvae revealed black bloat to be a form of forearm amelia caused from multiple organ system dysfunction (MODS). The animals typically near death were in the advanced stage of the bloat, with the forearms presumably unable to erupt because the internal organs visibly discernible

through the transparent skin appeared blackened. The death of the field population from black bloat was always in synchrony, affecting almost all tadpoles (Fig. 1B, C). 2) The specimen was not black, but bloated in the gastrointestinal region, which is discernible earlier in GS 19 (Fig. 2I-K, Fig. 5B). These bloated tadpoles with transparent skin were often the color of its internal fluid. The intensity of the red subcutaneous fluid colouration was dependent upon the concentration of the spray in the water of the site; the more concentrated and red-orange the spray water, the more red-orange the tadpoles appeared. The maximum fluid build-up in the stomach region resulted in lethality. Other abnormal red skin conditions were a red stained ventral epidermis, again the colour of the habitat water (Fig. 5E). A collected rare albino (Fig. 5D), a rare subcutaneous red anophthalmia (Fig. 5C) and a rare red patched tail with an internal gas bubble (Fig. 5F).

Stage 42-46 malformation (Fig. 6, 7; Table 2): the H artificial pond produced malformed metamorphs. Tadpoles grew to an abnormally gigantic GS 42 total size of 60 mm producing giant 21-23 mm SVL froglets. The same population commonly metamorphosed both oversized 21-23 mm and normal size 12-20 mm GS 46 (Fig. 6A). Normal brown, lime green intermingled with red-orange, yellow and rarely albino tadpoles and metamorphs were found in a single population. Some almost or recently metamorphosed kyphosis-emaciated and scoliosis frogs often showed abnormal colouration at metamorphosis (Fig. 6B-C). Another abnormality included one or both right and left forearm amelia (Fig. 6E-G). The tadpoles with both forearms amelia (Fig. 6E, G) had open wounds from failure of the slits in the body cavity to close. The larvae

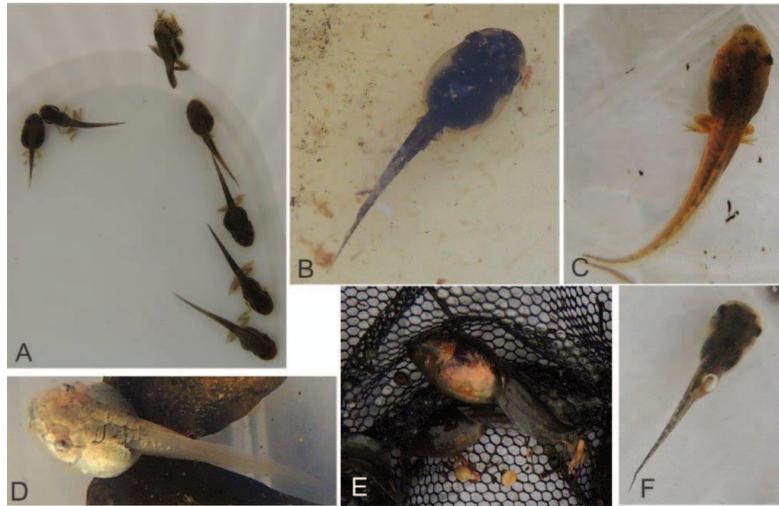


Figure 5. GS 25- GS 41 roadside drainage ditch. (A) GS 40-GS 42 bloat black. (B) GS 36 bloat clear with red subcutaneous fluid. (C) GS 39 one eye anophthalmia, red subcutaneous fluid (D) GS 25 albino. (E) GS 25 epidermal red discoloration. (F) GS 25 trapped bubble in tail.

unable to sprout both forearms had a strange swimming behaviour they swam on their back (Fig. 6G). Some tadpoles spent an abnormally extended period of time with their opercular sac extended without the forearm rupturing out (Fig. 6H). All post-metamorphic specimens from the site captured as GS 45 or GS 46 demonstrated spontaneous twitching of the limbs as older metamorphs neared adulthood.

Stage 46 malformation (Fig. 6-7; Table 2): Tail scoliosis (Fig. 6H-J) was a high frequency malformation found only at the untreated swimming pool in years 2012-2013 which produced both scoliosis or normal spine GS 46 metamorphose (Fig. 6I, J). The scoliosis tail appeared on the 5th day GS 25. There were different types of metamorph scoliosis (i.e., curvature of the spine) combined with or without total length growth retardation. The lethal scoliosis type occurred when the spine curved around the stomach with or without hind limb displacement (Fig 6I). In the common scoliosis cases (Fig. 6J) field captured GS 42 reared to GS 46 achieved a normal adult body size with sexual maturity evident in both genders, despite the formation of a large horn on the spine. The backbone rise with spine horn rapidly grew in height immediately after metamorphosis. Other abnormalities found at the untreated pool site were bloat (Fig. 6N), right forearm amelia (Fig. 6F), microcephaly-emaciation (lethal) (Fig. 6M), tumours on the tail (Fig. 6K) and black skin lesions (Fig. 6O).

The research effort resulted in 36,550 *O. septentrionalis* tadpoles examined in the field from 11 sites and found 25,136 abnormal ones, which is an abnormality incidence of 68.77 % (Fig. 8; Table 3). All sites in town had a high abnormality incidence regardless of treated or untreated water. The lowest abnormality average of 11.11 % occurred at site "CL" located on the outskirts of town, surrounded by forest (see Supplementary Materials Fig. S2E). The highest abnormality percentages came from the inner-city sites: HC 99.85 %, H container 98.9 %, H 75.29 %, H artificial pond 70.11 %, RP 57.36 % and RF 32.96 % (see Supplementary Materials Fig. S2A-E, G, J). The four non-spray control sites located inside the ENP Park and



Figure 6. GS 42-GS 46 H artificial pond abnormality. (A) GS 46, recently absorbed tail day 1 metamorphose. normal size 16mm (left), giant 21-23mm (right). (B) GS 46 kyphosis, emaciated, abnormal coloration, lethal. (C) GS 42 albino scoliosis. (D) GS 43, GS 45 lime green scoliosis giant (right) and brown normal size kyphosis (left). (E) GS 41 both forearm amelia with open wounds does not swim on back, lethal. (F) GS 42 right forearm amelia. (G) GS 42 both forearm amelia swims on back lethal. (H) GS 42 scoliosis, opercular sac abnormality. (I) GS 46 scoliosis hind limb displacement, growth retardation, lethal. (J) GS 46 scoliosis raised backbone with horn. (K) GS 41 tumor on tail. (L) GS 43 left hind limb amelia. (M) GS 46 kyphosis, emaciated, lethal. (N) GS 46 bloat clear, lethal. (O) GS 42 black spot on head.

Table 2. Individual animal count for each abnormality type collected at the H artificial pond (untreated) field site. Tadpole and metamorph abnormality type with count.

| Tadpole | Scoliosis | Forearm Amelia | Skin black Spot | Forearm Amelia Swim on Back | Bloat | Tail Tumour | Forelimb dev. | Others (Kphosis) | Normal |
|-------------|-----------|----------------|-----------------|-----------------------------|-------|-------------|---------------|------------------|--------|
| Total Count | 66 | 5 | 12 | 28 | 2 | 6 | 7 | 3 | 55 |
| Metamorph | Kyphosis | Scoliosis | Amelia | Microcephaly | Bloat | Black Spot | | | Normal |
| Total Count | 51 | 21 | 4 | 37 | 26 | 12 | | | 69 |

Table 3. Individual animal count totals for each field site. The % abnormality prevalence (left) and survival to metamorphosis (metamorph count) (right).

| Abnormal | Normal | % AB | Total Animal | Metamorph | Total Animal | % Metamorph | Raw number |
|---------------|--------|---------|--------------|-----------------|-------------------|-------------|-------------|
| RP | | | | RP met | RP total | | |
| 5799 | 4311 | 57.36 % | 10110 | 1 | 10110 | 0.01 % | 9.89E-05 |
| H | | | | H met | H total | | |
| 524 | 172 | 75.29 % | 696 | 84 | 696 | 12.07 % | 0.120689655 |
| HC | | | | HC met | HC total | | |
| 684 | 1 | 99.85 % | 685 | 3 | 685 | 0.44 % | 0.004379562 |
| H pond | | | | H pond met | H pond total | | |
| 129 | 55 | 70.11 % | 184 | 1762 | 3459 | 50.94 % | 0.509395779 |
| H containers | | | | H container met | H container total | | |
| 17608 | 196 | 98.90 % | 17804 | 13 | 17804 | 0.07 % | 0.000730173 |
| Cl | | | | CL met | CL total | | |
| 156 | 1248 | 11.11 % | 1404 | 11 | 1404 | 0.78 % | 0.007834758 |
| RF | | | | RF met | RF total | | |
| 117 | 238 | 32.96 % | 355 | 4 | 355 | 1.13 % | 0.011267606 |
| ENP/Ave Maria | | | | ENP/AM met | ENP/AM total | | |
| 0 | 2037 | 0.00 % | 2037 | 0 | 2037 | 0.00 % | 0 |

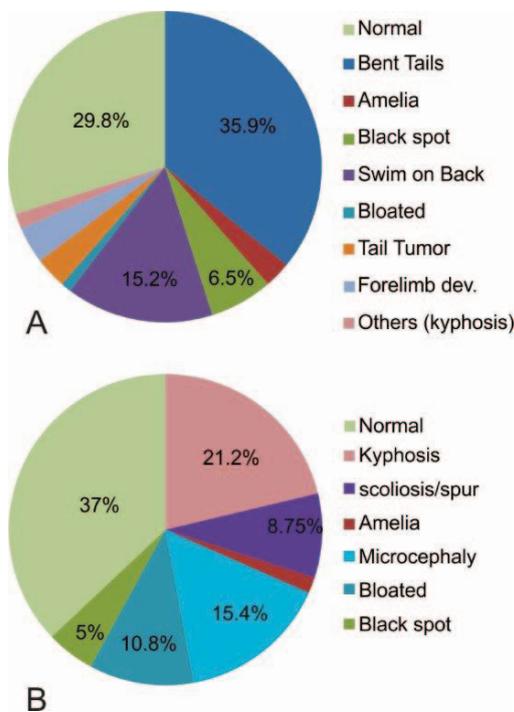


Figure 7. Pie Chart Graph of H artificial pond field site tadpole and metamorph abnormality types and proportions of each abnormality type (see Table 2). (A) Total tadpole abnormalities and proportion of each type. (B) Total metamorph abnormalities and proportion of each abnormality type.

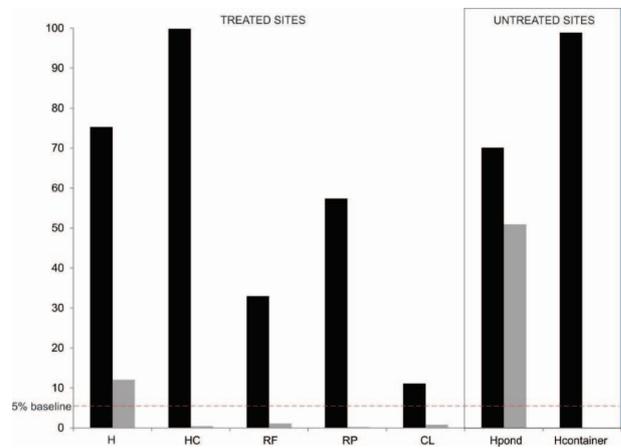


Figure 8. Bar Chart percentages of abnormal frogs (abnormality frequency) and percentages of number of metamorphs (survival to metamorphosis) for each in town field site H, HC, RF, RP, CL, H pond, H container for the field seasons 2012-2015 (see Table 3). The four control sites ENP (2 locations) & Ave Maria (2 locations) located in untreated insecticidal spray zones were not included in the Bar Chart having 0% abnormality. The H container site represents single clutch data.

Ave Maria community (see Supplementary Materials Fig. S2H, I, K, L) had no tadpole field abnormalities. A total of 1878 metamorphs were found at 8 out of the 11 sites, with almost all survival-to-metamorphosis from the untreated H artificial pond site (see Supplementary Materials Fig. S2F) in which 50.94 % had successfully mass metamorphosed into frogs. The insecticide treated sites (see Supplementary Materials Fig. S2A-E) had no more than 12.07 % survival to metamorphosis.

DISCUSSION

To know the entire biology of the frog population might mean a massive task for most scientists, considering many frogs are data deficient in biological data (Nori et al., 2018). This study couples *Osteopilus septentrionalis* embryonic and larval staging with a field study of a hotspot of frog abnormality. To identify a hotspot, it is necessary to know the entire biology and normal morphology for all developmental stages for the frog or of all frog species that frequent the site. The examination of eggs, embryos and all earliest developmental stages must be examined to find abnormality as it was this case in SW Florida.

Larval development in *O. septentrionalis* follows the standard table of Gosner (1960) for the Gulf Coast toad, *Incilius valliceps*, and extensive to most Orton's (1953) type-IV larvae. The duration of the larval period for the population studied here (around 19 days) is quite short compared with other populations and species described so far (e.g., Altig & McDiarmid, 2015). In fact, a previous description of larval growth of this species refers to larval growth as rapid as less than one month to the end of metamorphosis, at about 15-16 mm SVL (Meshaka, 1993, 2001). This data implies a fast rate of development and growth in the population under study here. Later in the lifecycle, by stage 42, specimens have attained their metamorphic size similar to most anuran species (e.g., Hall et al., 1997; Fabrezi, 2011). Also, significant post-metamorphic growth is needed to reach the minimum adult size in males (27 mm SVL) and females (45.0 mm SVL) (Meshaka, 2001). The metamorphic period, between stages 42 and 46, lasts 7 days which is the average for many species (e.g., Downie et al., 2004).

This study extends the geographic range and type of anuran abnormalities found in the United States to SW Florida (see USFWS, 2018). According to Duetet (2000) and Lannoo (2008) a hotspot area is any area with a 5 % or higher abnormality prevalence. By this definition, this SW Florida hotspot is unique in that it is the first report of a hotspot located in a suburban area in Florida with an unusually high 68.77% amphibian abnormality prevalence. The overall nationwide amphibian abnormality frequency average was low at 2 % abnormal, with three hotspots having a maximum abnormality frequency of 40 % (Reeves et al., 2013). An additional survey work reporting national hotspots maximum frequency increased in number from 3 to 96 hotspots with a maximum hotspot frequency of 25 % (Haas et al., 2018).

The USFWS survey (Reeves et al., 2013) differs from the SW Florida hotspot survey in site type. The National survey sampled frog populations in National park

wetlands without any anthropogenic disturbance. We sampled an anthropogenic disturbance site in a suburban area. USFWS found skeletal, eye abnormality and limb abnormalities. The limb abnormality portion of the national lab findings, which was over half of their findings, resulted an abnormality to be expected considering wetland trematode populations are known to naturally exist (Sessions & Ruth, 1990; Johnson et al., 1999, 2004). Since there were no trematodes in the suburban roadside ditch, we did not find most of the limb abnormalities reported by USFWS.

An abnormality we did find with considerably high prevalence, that USFWS found also in the park wetland, was the backbone abnormality scoliosis frog. At the SW Florida hotspot we found an 8.75 % prevalence of scoliosis while the national laboratory found a 12.6 % national frequency. SW Florida had 8.75 % prevalence of scoliosis in metamorphs with a 35.9 % larval prevalence (bent tail). This reduction in metamorph abnormality frequency (compared to a high of 35.9 % in larvae) could be attributed to the lethal types of scoliosis identified in this study and found only in the metamorph. The eye anophthalmia abnormality for both projects (national and local) was at background level frequency. The abnormalities presented by USFWS were scarce in type with some high frequency abnormalities. Our suburban hotspot exhibited numerous types of abnormality, very high frequency abnormalities are to be expected in consideration of the fact that we are dealing with a chemical agent with damage to the embryo in the very stage when the frog forms the head, body and tail.

It has been reported that mass malformations in amphibians correlate with global population declines (Alroy 2015; Whitfield et al., 2016). The known natural agents causing mass malformations said to be infective in nature include the microbial agent ranavirus (Teacher et al., 2010; Earl & Gray 2014), chytrid fungus *Batrachochytrium dendrobatidis* (Muths et al., 2003; Vredenberg et al., 2010) and a parasitic trematode agent (Johnson et al., 2002).

Malformations have also been linked to exposure to chemical agents in the field, and in the laboratory. Chemical agents are known to have direct effects on developing systems whereas infective agents such as trematodes do not (Lannoo, 2001). There are many experimental studies which demonstrate that chemical agents can disrupt normal embryonic development and induce congenital malformations (e.g., Osano et al., 2002; Hu et al., 2015). Lowcock (1997) demonstrated that abnormal genetic profiles were more frequent in green frog populations that had been exposed to pesticides. Epigenetic malformations occur when genes and gene expression are normal, but some deviation from expected environmental circumstances occurs at the time when genes are being exposed to cause the abnormality (Lannoo, 2001). Congenital malformations are defined as damage to the primary DNA sequence genome, either overall, or at a specific locus, or of the organ(s), tissue(s), or cell(s) resulting in the alteration of the developmental field (Hennekam et al., 2013).

Genomic analysis on abnormal *O. septentrionalis*

to look for genomic damage in order to classify the hotspot frog abnormality as congenital has not been performed. However, we do have data to hypothesize that our abnormalities are congenital, and inherited. The abnormality occurring at the hotspot in this study is at the embryonic stage during neural tube formation and early enough in development to indicate altered genes and gene loci. We also have found the same frog abnormalities in both the treated and untreated water on a repeat annual basis, which indicates the adults have been exposed to a chemical agent that is affecting their reproductive output. The reproductive organs are abnormal (Pratt Anzaldua, pers. obs). The types of gonadal abnormality found are classified as congenital in humans and associated with an increased gene copy number of the VAMP7 gene (Tannour-Louet et al., 2014).

A chemical-parasite agent combination has been recently proposed by USFWS as the leading cause for skeletal and eye abnormalities during metamorphosis (Haas et al., 2018). The recent USFWS (2018) report (Haas et al., 2018) claimed that the increase in hotspots nationwide is attributable to discovered oil well contamination at refuge sites. Interestingly, Florida larvicidal insecticides contain a petroleum distillate spreader. However, the abnormality found exclusively in the treated roadside ditch pool was black bloat, not scoliosis backbone abnormality, and not limb abnormalities, simply because the tadpoles could not even emerge their limbs to metamorph. In the treated water contaminated with the petroleum oil spreader, there was severe bloat which worsened with the increase in concentration and freshness of the spray. Conversely, skeletal scoliosis abnormality was not found in the treated water, so it is unlikely that scoliosis can be attributed to the petroleum oil. It is more likely that the bloat we see at the SW Florida hotspot is an abnormality likely attributable to oil poisoning because bloat is a symptom of oil poisoning in cattle exposed to oil fields (Osweiler, 2018).

Three studies support our hypothesis that a chemical agent or a combination of chemical agents is responsible for causing the Florida hotspot frog abnormalities. The same field tadpole mouthpart abnormalities comprising partial to complete absence of keratinisation of lower and upper tooth rows found in SW Florida suburban were recently found in SE Brazil (Navarro-Loano et al., 2018). The qPCR analysis of abnormal tadpoles detected no evidence of the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). In the laboratory, Sayim (2008) subjected GS 21 Marsh frog (*Rana ridibunda*) tadpoles to the organophosphate pesticide Malathion then photographed and described the same abnormalities as we describe: abnormal gut coiling, general edema, swimming on backs, and twitching. Bullfrog tadpoles in a constructed wetland located in Georgia which had received treated wastewater (Ruiz et al., 2010) were found with open wounds, due to failure of the slits to close after forelimb emergence, and tadpole scoliosis abnormalities.

It has been only recently recognised in herpetology the lack of studies assessing amphibian malformations and their causes in hotspot biodiversity areas (Sanchez-Domene et al., 2018). There have been even fewer

number of surveys for abnormal amphibians conducted in the suburban heavy mosquito control insecticidal spray zone areas. We have shown that the frequency of the frog abnormality in one small region in suburban Florida is alarmingly high at 68.77 % which may well indicate that all amphibians in Florida may be in particular trouble, when one considers that all of Florida has been subjected to the same pesticides. As such, this publication could be used as a tool for the identification of other malformation hotspots in Florida using *O. septentrionalis* as a sentinel species.

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Artificial reproduction using leuprolide acetate in the frog *Rana pipiens*

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Procuring offspring from captive animals can be important for research and conservation efforts. Yet, reliable methods for obtaining fertilised eggs are unavailable for many species. In this study, we examined the efficacy of one drug, leuprolide acetate, to induce reproduction (i.e. egg production, tadpole hatching) in the northern leopard frog, *Rana pipiens*. We found that leuprolide acetate successfully induced breeding and larval development in animals that were overwintered in the lab, but not in animals caught during the breeding season. These results indicate that leuprolide acetate can be successful in inducing breeding and fertilisation of frog eggs, but that its effectiveness might be contingent upon length of time in captivity and the animals having undergone an artificial overwintering period in the laboratory prior to induction. Artificial breeding success is species and context dependent; therefore, identifying additional methods effective across taxa will help support species in need of management intervention.

Keywords: Amphibian; Amphibian reproduction; Assisted reproductive technologies; Breeding; Hormone induction; Leuprolide Acetate; Spawning

INTRODUCTION

Assisted reproductive techniques can play an important role in the management of declining or endangered species. Developing and refining protocols for captive breeding can facilitate basic wildlife research, enable effective management of captive populations, and improve reintroduction and management outcomes (Wildt, 2000, Andrabi & Maxwell, 2007). Amphibians are currently experiencing dramatic global declines in biodiversity (Skerratt et al., 2007). Because many species of amphibian do not breed easily in captivity, assisted reproduction methods are being developed to aid in the conservation of some declining species (Kouba et al., 2012, Clulow et al., 2014). Hormone treatment is often required to induce reproduction in captive animals, and although a number of drug treatments have been shown to be effective in inducing fertilisation, their effectiveness can be highly species and environment dependent (Trudeau et al., 2010, Silla & Roberts, 2012).

In vertebrates, ovulation and sperm production requires luteinizing hormone, and gonadotropin-releasing hormone (GnRH) is the primary stimulator of luteinizing hormone production. Artificial reproduction treatments often use GnRH or luteinizing hormone analogues to promote ovulation and sperm release (Clulow et al., 2014, 2018). A luteinizing hormone-like compound called human chorionic gonadotropin (hCG) can be effective in some frog species, but this hormone has been much more variable in its ability to induce ovulation and/or sperm production in

other amphibians (Mann et al., 2010, Clulow et al., 2018). Another commonly used induction method involves injection of a combination of a GnRH-agonist compound and a specific catecholamine dopamine receptor, both of which have been designed to block the inhibitory effects of dopamine that enables the release of gametes in vertebrates. One catecholamine dopamine receptor that has been effective in inducing reproduction in a number of frog species is metoclopramide hydrochloride (Trudeau et al., 2010). However, GnRH agonist compounds alone, without a coupled specific catecholamine dopamine receptor, have also been successful in inducing reproduction (Vu et al., 2017). Leuprolide acetate is a GnRH agonist that has been used successfully to induce reproduction in a number of frog species (Byrne & Silla, 2010; Trumbo, 2015, Clulow et al., 2018). Leuprolide acetate is the medical compound in the drugs Lupron (Tap Pharmaceuticals) and Lucrin (Abbott), which are used in treating prostate cancer and endometriosis, and for in vitro fertilisation in humans (Chetkowski et al., 1989; Dlugi et al., 1990; Persad, 2002).

The purpose of this study was to trial the use of leuprolide acetate as a method of inducing reproduction in *Rana pipiens*, a species that is often maintained in captivity for research and educational purposes (e.g. teaching dissections). Captive animals used in scientific experiments and for education are often wild caught, even if purchased through a supplier (with large scale suppliers shipping animals across the United States). Developing alternative methods for captive breeding would reduce stress on wild populations, and promote

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the establishment of fully captive colonies for science and educational purposes. Further, *R. pipiens* populations are declining in parts of their native range. Although some populations are stable, captive rearing and reintroduction efforts are employed in areas where populations have declined, with active reintroductions for 35 years, and almost 8,000 animals released per year (Randall et al., 2016). In captive *R. pipiens*, hormone inductions are required, because males and females will not successfully mate without intervention (Trudeau et al., 2010; Vu et al., 2017). For *R. pipiens*, the combination of a GnRH agonist plus metoclopramide hydrochloride, as well GnRH agonist alone, have been shown to induce reproduction with a fertilisation rate of between 70 and 90 %; however, success often varies with the timing of induction and can also depend upon captive conditions prior to induction (Trudeau et al., 2010; Vu et al., 2017). Therefore, in this study we, 1) tested an alternative drug, leuprolide acetate, which has been trialled in many other amphibian species (reviewed in Clulow et al., 2018), but not in *R. pipiens*, and 2) compared the drug's ability to successfully induce reproduction and lead to the hatching of tadpoles in animals that were captured from the wild and housed under different conditions. Wild individuals were caught during the breeding season (in the early spring upon ice melt following hibernation) from three different populations, maintained in captivity for a short period, and injected with leuprolide acetate to induce reproduction at the same time. Next, we tested the efficacy of housing animals in captivity for an extended period of time, artificially overwintering them, and inducing reproduction after emergence from overwintering. The implications of this trial, specifically with using animals from different populations, are to determine 1) if and how animals from different populations and different natural timing of reproduction can be induced at the same time, and 2) to determine if leuprolide acetate is effective at inducing reproduction in *R. pipiens*.

METHODS

Animal Husbandry

The animals were wild-captured by hand during the early spring breeding season just following their natural hibernation in 2017 from three locations: Crosby Township, Ohio (February 24: one female, four males), Linesville, Pennsylvania (March 26 & 28: five females, four males) and Milton, Vermont (May 17: three females, five males). A second group of wild caught animals were collected in the autumn of 2017 from Shelburn, Vermont (October 14, 21 & 22: four females) and Linesville, Pennsylvania (October 11: three females, six males).

After collection and before hormone treatment, animals were housed in the laboratory in individual enclosures (36 x 21 x 25 cm) containing 1 L aged tap water and tilted to provide both aquatic and terrestrial habitats. A hide made of PVC pipe cut in half length wise (8 cm long x 3 cm high) was placed in each enclosure. When not breeding, *R. pipiens* tends to spend more time in the terrestrial environment, and 1L or less per individual is sufficient (Boice & Williams, 1971; Tennessen et al., 2009). Animals were fed vitamin-dusted crickets ad libitum and

water was changed every five days. The animals were maintained at room temperature (18 to 23 °C) with a light:dark cycle of 14:10 h, and monitored daily for general health.

Breeding Mesocosms

Mesocosms (Rubbermaid stock tanks, 189 L capacity) were set up indoors, filled with 70 L of aged tap water, covered with screen and provisioned with a bundle of sticks (for attachment of egg masses). The mesocosms had a step on the inside, which the animals used as a terrestrial habitat, and a floating plastic platform (25 x 17 cm) was also provided. The air temperature was maintained at 16 to 18 °C, which is within the range of temperatures for *R. pipiens* breeding in nature (Gilbert et al., 1994; Trudeau et al., 2010). Half water changes were performed every 10 days, and animals in the mesocosms were fed adult crickets ad libitum every five days. After each trial was complete, animals were returned to their individual enclosures. Number of animals per mesocosm per experiment varied because we wanted to keep populations separate. Mesocosms were checked at least once per day and egg masses were removed as described below.

Hormone Treatment

After acclimation, the animals were treated with leuprolide acetate in the evening, 2 h before sunset. Males and females were injected intraperitoneally using a 26 gauge needle with 0.2 mg of leuprolide acetate (LupronDepot®, Tap Pharmaceuticals), diluted in sterile APBS, for an injection volume of 500 µL per animal (Bowcock et al., 2013; Trumbo, 2015). Twenty-four hours following the first injection, all females were treated a second time with 0.1 mg of leuprolide acetate in APBS for an injection volume of 250 µL per female. The leuprolide acetate stock solution was stored at -20 °C between trials. After injection animals were checked daily, and the egg masses they produced were transferred to new containers (36 x 21 x 25 cm), filled with 5 L aged tap water, and monitored for development for at least seven days. Half water changes were conducted every five days. Egg mass success was determined by observed egg hatching. Animals remained in the breeding mesocosms for seven days.

No hormone negative controls were included in this study because in *R. pipiens*, it is generally understood that under captive conditions animals will not successfully mate without a hormone induction (Trudeau et al., 2010, 2013; Vu et al., 2017).

Induction in Animals Caught During the Breeding Season

Animals were captured from the wild during their spring breeding season (February-May), transported to the lab, and maintained in an environmental chamber (Conviron) at 8 °C prior to hormone treatment (Ohio animals for 12 wk, Pennsylvania animals for 9 wk, and Vermont animals for 1 wk). The purpose of the delay for some populations was to induce all animals together, for experimental purposes, such as a common garden experiment of several populations. Animals were warmed slowly to 16 °C over seven days (1.1 °C per day), and placed inside the breeding mesocosms on May 23, 2017. Animals were housed communally by population with a maximum of 6

animals per mesocosm (similar to male to female ratios of Trudeau et al., 2010; Vu et al., 2017): Ohio animals in one mesocosm, Pennsylvania animals in two mesocosms, Vermont in one mesocosm (Supp. Table 1). The animals were allowed to acclimate inside the mesocosms for at least 36 h before hormone treatments began. Animals were then injected with leuprolide acetate, following the protocol described above.

Artificial Overwintering and Induction

Animals collected from Pennsylvania and Vermont were used in this experiment. Some animals were collected in the autumn of 2017 ($n = 10$), and the others were from the previous experiment (collected in the spring and held in captivity for 10 to 12 mo, $n = 16$). All animals were housed individually, as described above, prior to mesocosm acclimation and hormone treatment. Animals were overwintered at 4 °C for 8 wk (December to February) in the lab, similar to what has been described previously for captive breeding in *R. pipiens* (Trudeau et al., 2010; 2013). For overwintering, animals were placed in an environmental chamber (Conviron) and the air temperature in the chamber was slowly decreased from 18 °C to 4 °C over a period of nine days (1.5 °C / day). During this period, the water was changed once but the animals were no longer fed, with feeding ending 3 days before cooling began. Once the temperature reached 4 °C, the animals were moved to smaller enclosures (25 x 17 x 10 cm) filled with 2 L of pre-chilled aged tap water, which was enough to ensure that the animals remained submerged. The animals were kept in the dark, and half water changes were conducted with pre-chilled aged tap water every seven to 10 days (Lillo, 1980). Immediately before water changes, oxygen levels in three haphazardly chosen enclosures were monitored (using a YSI ProODO Handheld Oxygen Meter) to ensure levels remained above 70 % dissolved oxygen, near the optimum pressure for O₂ absorption efficiency of ranid frogs and well above hypoxic conditions (Tattersall & Ultsch, 2008). After 8 wk of overwintering, the air temperature in the environmental chamber was gradually increased from 4 °C to 16 °C over nine days (1.5 °C / day). During this time the animals remained in the same enclosures, but with 0.5 L of water, and with the enclosures tilted to provide aquatic and terrestrial habitats. We began feeding again on day five of this gradual warming period. After the warming period, animals were transferred to breeding mesocosms (with ratio as close as to 1 female and 1 male per mesocosm (e.g. Silla et al., 2018) as possible while maintaining population and collection time separation), and induced with leuprolide acetate following a three day acclimation period, as described above.

Ethics Approval

This research was conducted under the University of Pittsburgh Institutional Animal Care and Use Committee protocol IML-17091291-9 "Effects of climate change on host-pathogen interactions in chytridiomycosis." The animal collection was conducted under permits from Pennsylvania Fish and Boat scientific collection permit number 2017-01-0177, Ohio Division of Wildlife Wild animal permit number 18-149, and Vermont Fish and Wildlife Department scientific collection permit number SR-2016-17.

Analyses

We compared the number of females that produced egg masses that successfully hatched tadpoles to those that produced either no egg mass or an inviable egg mass across two treatment groups using a 2 x 2 Pearson's chi-squared test in SPSS (v21, IBM Corp. Armonk, NY). Egg mass success was operationally defined by the production of tadpoles. Egg masses that did not develop to hatching were considered unsuccessful.

The first analysis compared the production of viable egg masses between the groups of animals that were caught in the spring and induced directly after the breeding season with those animals that were induced after being artificially overwintered. In this analysis we compared all females that produced completely viable or partially viable (if some but not all of the eggs in the egg mass hatched) egg masses to those that produced either a completely inviable egg mass or no egg mass at all.

The second analysis involved only the animals that experienced an artificial overwintering in the lab, and we compared the proportion of females that produced completely viable egg masses (the whole egg mass hatched) between animals captured during the spring (breeding season) or autumn. Population of origin was not accounted for in our statistical analyses due to insufficient sample size.

RESULTS

The results of this experiment demonstrate that *R. pipiens* can be successfully bred in captivity using leuprolide acetate, but only under certain conditions. In the animals caught during their breeding seasons (February, March and April respectively), they appeared reproductively active ($n = 21$), with all females ($n = 9$) gravid (in that they visibly looked wider in their midsection than the males) at the time of capture. Following induction, one female (11.1 % of females; Fig. 1a; Supp. Table 1; had been held in captivity for 12 weeks) produced a small, partial clutch, which was partially successful (only some eggs hatched), and no female produced a fully successful egg mass. Some females ($n = 5$) released their eggs over a period of days/weeks during captivity prior to induction (all females collected from Pennsylvania).

In animals that were overwintered in the lab ($n = 29$, 15 females, 14 males), 60.0 % of the females produced egg masses (9/15), and all produced some tadpoles, but 66 % of those (6 of 9 total egg masses) were successful at producing a large proportion of tadpoles (Supp. Table 1). The proportion of females that produced a successful egg mass after hormone treatment was significantly higher when the animals were artificially overwintered in the lab compared with those collected during their spring breeding season (Fig. 1a; Pearson's: $n = 24$, $\chi^2_1 = 5.531$, $P = 0.033$).

When considering only animals that were artificially overwintered in the lab prior to hormone treatment, there was no significant difference in the proportion of females producing a viable egg mass between autumn and spring collected animals (Fig. 1b; Pearson's: $n = 15$, $\chi^2_1 = 3.616$, $P = 0.119$). 75 % of the females (6/8) that were collected in the spring and overwintered produced an egg mass,

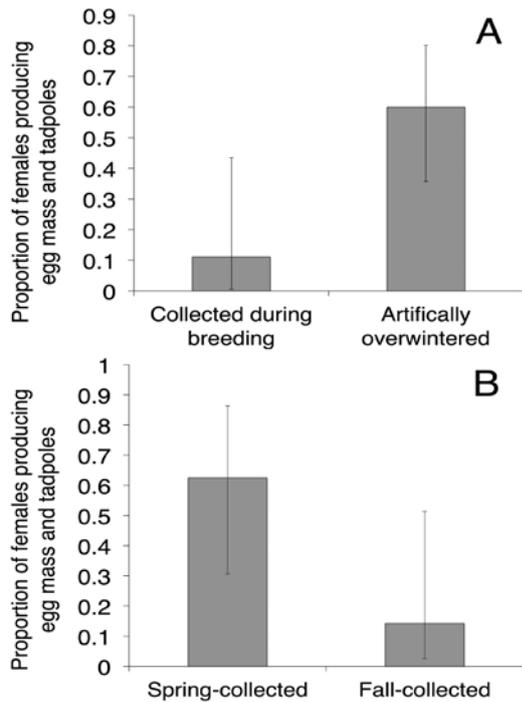


Figure 1. The proportion of females that produced viable egg masses across experiments. Comparisons include A) the proportion of egg masses that were fully or partially viable collected during the breeding season versus those that were artificially overwintered and B) the proportion of fully viable eggs laid from frogs collected in the spring versus autumn and overwintered in the lab. The error bars represent 95 % confidence intervals.

while of the autumn collected and overwintered animals, 43 % (3/7) of the females produced an egg mass. Only one of the egg masses from the autumn-collected animals hatched tadpoles (Supp. Table 1, Fig 1b).

The animals we collected in the spring and held in captivity for nearly one year were large, reproductively mature adults ($n = 17$; range = 35 to 63 g, mean \pm sd = 47.65 ± 9.06 g) at the time of induction; females were gravid after overwintering, and males had visibly defined secondary sexual characteristics that indicate breeding status such as enlarged vocal sacs and darkened nuptial pads. However, most animals collected in the autumn (11 of 13) were small (likely young-of-the-year from 2017; 11 to 27 g, mean \pm sd = 18.73 ± 4.41 g at time of induction). The small females did not appear gravid at the time of induction, but the males had darkened nuptial pads and visible vocal sacs. Two of the females collected in the autumn were large (60 and 64 g) and appeared gravid.

All but two (80 %) of the large adult females that were overwintered (> 35 g at time of induction) produced a full egg mass, and one of those two females produced a handful of viable eggs but not a whole egg mass (see footnote on Supp. Table 1). It should be noted that one small female (19 g at time of induction, collected in the autumn) produced a full egg mass that successfully hatched tadpoles, although the size of the mass was much smaller than those produced by the larger females.

One animal died during overwintering in the lab (a male that was caught in the spring). This male frog survived

three weeks after entering overwintering conditions. Another male that was collected in the spring died during the acclimation period inside the breeding mesocosms (two days after entering the breeding mesocosm). The causes of these deaths are unknown.

DISCUSSION

Assisted reproductive techniques can be useful for wildlife conservation, particularly in collaboration with captive breeding programs for declining species (Wildt, 2000; Andrabi & Maxwell, 2007). They can also be useful in basic scientific research for applications that require the production of viable embryos at a specific time. While there are a number of hormone induction methods published for use in amphibian species, no one method works for all species. The use of leuprolide acetate had not been reported previously in *R. pipiens*, but this treatment has been used with success in many other amphibians (Silla & Roberts, 2012; Trumbo, 2015; Clulow et al., 2018). In this study we compared the number of females producing egg masses that develop to tadpoles among groups of animals that had experienced different durations of captivity prior to treatment with leuprolide acetate. We were unsuccessful in procuring viable egg masses from animals collected during their spring breeding season (when females were naturally gravid) and maintained for a period of one to 12 weeks in the lab, but achieved higher success in induction of animals after they had been in captivity for an extended period of time.

We had little success in procuring tadpoles from animals we collected during their breeding season. Interestingly, other published accounts describe higher success rates for same-season induction using other hormone treatments. *Rana pipiens* have previously been collected upon emergence from their overwintering habitat, and maintained under laboratory conditions for up to one month prior to induction using a GnRH-agonist compound and a specific catecholamine dopamine receptor antagonist, with high reproduction success after induction, between 70 and 90 % fertilisation success (Trudeau et al., 2010; Vu et al., 2017). One of the aims of this study was to investigate if induction could be delayed in animals caught during their breeding season so that individuals from different populations could be induced together, a useful tool for certain experimental designs. Our study demonstrates that delayed induction using leuprolide acetate was unsuccessful if animals were collected and induced while naturally gravid.

The failure for induced breeding in the spring-collected animals could be due to the extended stay in captivity prior to hormonal induction. In two of the three populations tested we housed the animals in captivity for up to eight weeks longer than previously published accounts (Trudeau et al., 2010; Vu et al., 2017), and the females had been gravid for much longer than they would have been in nature in those populations (Zenisek, 1963). Some of the gravid females we collected began to deposit eggs in their individual enclosures prior to hormone induction treatment. When a healthy gravid female is unable to successfully mate, she might either arrest egg development or reabsorb the eggs (Kouba et al., 2012).

Perhaps these females' eggs had become too developed to be successfully arrested or reabsorbed, causing the females to release them in order to avoid becoming egg-bound, which can be fatal (Kouba et al., 2012). Perhaps these animals would be successful at producing offspring if they had been induced earlier, rather than waiting for the last population to emerge for breeding. However, it should be noted that none of the animals from the last population we collected (Vermont) successfully produced egg masses during induction, and these animals were induced within two weeks of capture, which is similar to other previously conducted studies (Trudeau et al., 2010; Vu et al., 2017). One possible explanation for this failed induction attempt is that these animals might have appeared gravid, but might not have been gravid enough to reproduce. We did not perform an ultrasound to verify the gravid status of the females (Calatayud et al., 2018; Graham et al., 2018), but because they were captured during active aggregate breeding it was assumed that females were present in the pond for the purpose of laying eggs.

Further, the male to female ratio within the mesocosms could have limited success of induction, as the only mesocosm with one female to four males was the one that laid a partial egg mass. We had tried to follow the ratio of males to females as had been successful for this species previously (Trudeau et al., 2010; Vu et al., 2017), but it is possible that fewer females to a mesocosm or a higher male to female ratio might have improved success. While the sample sizes are small within this experiment, our results indicate that animals collected in the spring and induced with leuprolide acetate soon after are less likely to successfully reproduce. To limit the effects of sex specific competition, we reduced the density for our next induction trial.

After our attempts to breed spring-collected animals failed, we maintained those animals in captivity and captured more animals to test for an effect of collection time and overwintering on induced breeding success. We were more successful in obtaining egg masses that hatched tadpoles from animals that had been artificially overwintered in captivity prior to hormone treatment than from animals that had been collected during the breeding season, and animals from both populations (Pennsylvania & Vermont) successfully produced tadpoles (Supp. Table 1). Nine egg masses were produced by animals that were overwintered in the lab (60 % of the females produced an egg mass), and 66 % of those egg masses produced a large proportion of tadpoles. Production of egg masses that are only partially successful at hatching tadpoles is a common problem in captive breeding colonies. Failed development/fertilisation of some or all eggs in a clutch can be caused by a number of factors, including poor adult nutrition, lack of environmental stimuli, and stress associated with captivity (Kouba et al., 2012). Captive animals are often less reproductively successful than their wild counterparts and some reproductive failure is to be expected (Kouba et al., 2012).

Some of our autumn-collected animals were likely young of the year, which might explain why we saw fewer egg masses produced following our induction with leuprolide acetate than other trials have reported for induction in *R. pipiens* (Trudeau et al., 2010; Vu et al.,

2017). In those young animals we found that the male *R. pipiens* exhibit all the signs of sexual maturity (i.e., have dark thumbs, large forearms and vocal sacs). However, female *R. pipiens* do not typically breed until their second year of life (Gilbert et al., 1994). In our study we found that one small female (19 g) produced an egg mass that successfully hatched tadpoles, indicating that some females might become sexually mature in their first year of life. Perhaps in an effort to optimise breeding success (both egg size and number of eggs), *R. pipiens* females wait until they reach a larger size to begin breeding (Tavecchia et al., 2001). Female size is correlated with reproductive success (i.e. number of viable offspring) in a wide range of taxa, including frogs (Tejedo, 1992; Jorgenson et al., 1993). Artificial reproduction of wild caught animals housed under captive conditions is important for a wide variety of scientific pursuits. *Rana pipiens* is a species that is declining in parts of its range, and therefore, artificial reproduction in this species might be important for conservation purposes (Randall et al., 2016). Trialling a new induction method for *R. pipiens*, we found that adults maintained in captivity and artificially overwintered prior to leuprolide acetate treatment were more successful in producing offspring compared to those caught during the breeding season, held in captivity and induced soon after. Using this combination of leuprolide acetate and captive management, we were able to stimulate reproduction in the majority of females, and the majority of the egg masses they produced developed to hatching. While there are other hormone therapies that have reported success in inducing reproduction in amphibians, it is important to explore multiple methods, because as our study shows, timing of induction and captive conditions might affect success. Although our study trialled hormonal induction procedures only in *R. pipiens*, the success of leuprolide acetate in this and other species (Silla & Roberts, 2012; Clulow et al., 2018) indicates that use of this hormonal induction method could be beneficial to other amphibian taxa in need of artificial reproductive technologies for conservation intervention as well.

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