Universitat
de Girona

# UNDERSTANDING THE ECOLOGICAL SUCCESS OF TWO WORLDWIDE FISH INVADERS (*Gambusia holbrooki* AND (Gambusia affinis)

## Pao SREAN

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**Ph.D. Thesis**

**Understanding the Ecological
Success of Two Worldwide Fish Invaders
(*Gambusia holbrooki* and *Gambusia affinis*)**

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DOCTORAL THESIS

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DOCTORAL PROGRAMME IN EXPERIMENTAL SCIENCES AND
SUSTAINABILITY

Supervised by

Prof. Dr. Emili GARCÍA-BERTHOU

Institute of Aquatic Ecology, University of Girona

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El Dr. Emili García-Berthou, catedràtic d'ecologia de la Universitat de Girona,

DECLARA:

Que el treball titulat "Understanding the ecological success of two worldwide fish invaders (*Gambusia holbrooki* and *Gambusia affinis*)", que presenta Pao Srean per a l'obtenció del títol de doctor, ha estat realitzat sota la meva direcció.

I, perquè així consti i tingui els efectes oportuns, signo aquest document.

Emili García-Berthou, Professor of Ecology
Institute of Aquatic Ecology & Dept. Environmental Sciences
University of Girona
Personal web page: http://invasiber.org/EGarcia/

Girona, 7 / gener / 2015

*Dedicated to my wife Sinet RIEN and my daughter Paomonika SREAN*

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# List of Abbreviations

AgriLand : Per cent area of agricultural land

AIC : Akaike's information criterion

ANCOVA : Analysis of covariance

Chl-$a$ : Chlorophyll $a$

CI : Confidence interval

cm : Centimetre

DIAS : Database on Introduction of Aquatic Species

DEP : Depth

DMO : Density of mosquitofish

DO : Dissolved oxygen

DTA : Density of target species

FAO : Food and Agriculture Organization

FAS : Factorial aerobic scope

Forests : Per cent area of forest coverage

Exports : Export as percentage of gross domestic product

GAM : Generalized additive model

GBIF : Global Biodiversity Information Facility

GDPC : Gross domestic product per capita

GISD : Global Invasive Species Database

ISSG : Invasive Species Specialist Group

IUCN : International Union for Conservation of Nature

MMR : Maximal metabolic rate

MR : Metabolic rate

LAT : Absolute latitude

lg_FrostDays : $\log_{10}$ of frost day frequency

lg_GDPC : $\log_{10}$ of gross domestic product per capita

lg_MeanTemp : Mean temperature

lg_MinTemp : Minimum temperature

lg_Precip : Log base 10 of precipitation

N : Nitrogen

$O_2$ : Oxygen

P : Phosphate

| | |
|---|---|
| PopDensity | : Human population density |
| RE | : Random-effect |
| REML | : Restricted maximum-likelihood |
| RMR | : Resting metabolic rate |
| SD | : Standard deviation |
| SE | : Standard error |
| SL | : Standard length |
| SS | : Suspended solid |
| TEM | : Temperature |
| TL | : Total length |
| UK | : United Kingdom |
| USA | : United States of America |
| WW | : Wet weight |

# List of Figures

# List of Tables

# Table of Contents

# Abstract

Biological invasions are currently a huge, global environmental issue in freshwater ecosystems. The mosquitofishes *Gambusia holbrooki* and *G. affinis* are freshwater poeciliid fishes native to North America that have been introduced worldwide since the early 1900s and are among the world's most invasive fish. They were regarded as three separate species and later as two subspecies of a single species before a genetic study in 1988, so their distribution is unclear. This thesis aims to contribute to the understanding of the invasive success of these two invasive fishes. We reviewed four Internet databases and the literature to clarify their introduction history and geographical distribution, establish their introduction routes, and analyse predictors of their invasive success. The four databases reviewed contain many clear errors and we estimate that *G. holbrooki* has established in ca. 49 countries and *G. affinis* in 44. For predicting introduction probability and establishment success of mosquitofish, the best explanatory variables among those available were related to climate, such as mean temperature or latitude, whereas for the probability of donating mosquitofish, economic drivers such as the intensity of exports and gross domestic product per capita were more important. A meta-analysis of the published evidence of ecological impacts of mosquitofishes shows a number of points, namely that the overall impact is: i) similar for the two species; ii) clear and strong for fish, macroinvertebrates, amphibians, and some zooplanktonic groups (such as copepods and rotifers) and more variable for taxa at lower trophic levels and for ecosystem features; iii) general for the number of aggressive acts received and decreases in density and biomass but more variable for other response variables such as size structure, life history traits, or other behavioural traits; and iv) highly heterogeneous and context-dependent on a number of features such as the target species involved, the experimental setting (depth of the study system, density of fish used), and environmental factors such as temperature. We estimated the mean critical swimming speed ($U_{crit}$) of *G. holbrooki* as 14.11 cm s$^{-1}$ (range = 4.85–22.26), which is lower than for many other fish of similar size and confirms that this species is limnophilic and its invasive success might be partially explained by hydrologic alteration. However, we demonstrate that $U_{crit}$ and maximal metabolic rate vary markedly with fish size and sex, with males having much higher values for the same weight, and thus probably being more resistant to strong water flows.

# Resum

Les invasions biològiques són actualment un enorme problema ambiental global, especialment als ecosistemes d'aigua dolça. Les gambúsies *Gambusia affinis* i *G. holbrooki* són peixos d'aigua dolça de la família dels pecílids, natius d'Amèrica del Nord, que es van introduir a tot el món des de principis del segle XX i es consideren entre les espècies de peixos més invasors. Al principi van ser considerats com a tres espècies diferents i més tard com dues subespècies d'una mateixa espècie, abans d'un estudi genètic a l'any 1988, pel que la seva distribució no està clara. Aquesta tesi té com a objectiu contribuir a millorar la comprensió de l'èxit invasor d'aquests dos peixos. Es van revisar quatre bases de dades d'Internet i la bibliografia per aclarir la seva història d'introducció i distribució geogràfica, establir les rutes d'introducció i analitzar els predictors del seu èxit invasor. Les quatre bases de dades revisades contenen molts errors clars i estimem que *G. holbrooki* s'ha establert a uns 49 països i *G. affinis* a uns 44. Per predir la probabilitat d'introducció i l'èxit de l'establiment de les gambúsies, les millors variables explicatives entre les disponibles van ser variables climàtiques com latitud o temperatura mitjana, mentre que per a la probabilitat de donar gambúsies van ser més importants factors econòmics, com la intensitat de les exportacions i el producte intern brut per càpita. Una meta-anàlisi de l'evidència publicada dels impactes ecològics de les gambúsies mostra una sèrie de punts, principalment que l'impacte global és: i) similar per a les dues espècies; ii) clar i fort per a peixos, macroinvertebrats, amfibis, i alguns grups de zooplàncton (com copèpodes i rotífers) i més variable per als tàxons de nivells tròfics més baixos i a nivell d'ecosistema; iii) general per al nombre d'agressions rebudes i la disminució de la densitat i la biomassa, però més variable per altres variables de resposta com l'estructura de mides, trets de cicle vital, o altres trets de comportament; i iv) altament heterogeni i dependent del context per una sèrie de característiques com ara espècies objectiu en qüestió, condicions experimentals (profunditat del sistema d'estudi, densitat de peixos utilitzada) i factors ambientals com la temperatura. Hem estimat la velocitat crítica de natació mitjana ($U_{crit}$) de *G. holbrooki* com 14.11 cm s$^{-1}$ (rang = 4.85–22.26), que és inferior a la de molts altres peixos de mida similar i confirma que aquesta espècie és limnòfila i el seu èxit invasor parcialment s'explica per l'alteració hidrològica. No obstant això, vam demostrar que $U_{crit}$ i la taxa metabòlica màxima varien notablement amb la mida i el sexe dels peixos, amb valors molt més alts als mascles que a femelles del mateix pes, els quals deuen ser per tant menys vulnerables a cabals forts.

# Resumen

Las invasiones biológicas representan actualmente un problema ambiental global enorme, especialmente para los ecosistemas de agua dulce. Las gambusias *Gambusia holbrooki* y *G. affinis* son peces de agua dulce de la familia de los poecílidos, originarios de América del Norte, que fueron introducidos en todo el mundo a partir de principios del siglo XX y se consideran entre las especies de peces más invasoras. Inicialmente fueron tratadas como tres especies separadas, y luego como dos subespecies de la misma especie, antes de un estudio genético del 1988. Por ello su distribución no está clara. Esta tesis pretende contribuir a mejorar la comprensión del éxito invasor de estas dos especies. Revisamos cuatro bases de datos de internet y la bibliografía para aclarar la historia de su introducción y la distribución geográfica, determinar las rutas de introducción y analizar los predictores de su éxito invasor. Las cuatro bases de datos revisadas contienen muchos errores claros. Estimamos que *G. holbrooki* se ha establecido en unos 49 países y *G. affinis* en unos 44. Para predecir la probabilidad de introducción y el éxito de aclimatación de las gambusias, las mejores variables explicativas entre las disponibles fueron variables climáticas como la latitud o la temperatura media, mientras que para la probabilidad de donar gambusias, factores económicos como la intensidad de exportaciones y el producto interno bruto per cápita fueron más importantes. Un meta-análisis de la evidencia publicada sobre el impacto ecológico de las gambusias muestra una serie de puntos, principalmente que el impacto global es: i) similar para las dos especies; ii) claro y fuerte para peces, macroinvertebrados, anfibios, y algunos grupos de zooplancton (como copépodos y rotíferos) y más variable para taxones de niveles tróficos inferiores y a nivel de ecosistema; iii) general para el número de agresiones recibidas y la disminución de la densidad y la biomasa, pero más variable para otras variables de respuesta como la estructura de tallas, rasgos de historia de vida u otros rasgos de comportamiento; y iv) altamente heterogéneo y dependiente del contexto en una serie de características tales como especies objetivo en cuestión, las condiciones experimentales (profundidad del sistema de estudio, densidad de peces utilizados) y factores ambientales como la temperatura. Estimamos la velocidad crítica de natación media ($U_{crit}$) de *G. holbrooki* como 14.11 cm s$^{-1}$ (rango = 4.85–22.26), que es inferior a la de muchos otros peces de tamaño similar y confirma que esta especie es limnófila y su éxito invasor se explica parcialmente por la alteración hidrológica. Sin embargo, demostramos que $U_{crit}$ y la tasa metabólica máxima varían marcadamente con el

tamaño y el sexo de los peces; los machos tienen valores mucho más altos que hembras del mismo peso, siendo por tanto probablemente menos vulnerables a caudales elevados.

# General Introduction

*Biological invasions in fresh waters*

Biological invasions are a serious problem in freshwater ecosystems and represent huge ecological and economic costs worldwide (Ricciardi & MacIsaac 2011; Fausch & García-Berthou 2013). Invasive species affect the distribution, abundance and reproduction of many native species (Strauss *et al.* 2006) and they have caused the decline and extinction of native freshwater fish species (Williams *et al.* 1989; García-Berthou *et al.* 2005). The process of invasion of non-native species outside its native range is divided into the following three main stages (Richardson *et al.* 2000; Kolar & Lodge 2001, Fig. 1):

i)   INTRODUCTION: species entrained in transport pathway into places where they are not indigenous,

ii)  ESTABLISHMENT (naturalization): the introduced species survive in places where they are not native and they have naturally self-sustaining population,

iii) INVASION: population growth and spread of the introduced species.
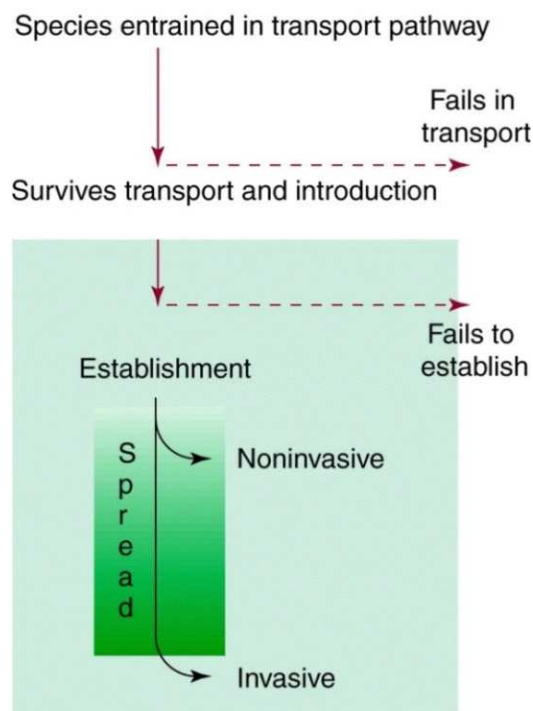


Figure 1: Transitions that non-native species must overcome to become invasive species (from Kolar & Lodge 2001).

In freshwater ecosystems, biological invasions have been increasing rapidly, and most invasions are irreversible and control is costly, therefore prevention of future invasions should be prioritised (Fausch & García-Berthou 2013). For example, non-native freshwater fish are distributed worldwide and have invaded all continents (Fig. 2; Leprieur *et al.* 2008). Freshwater invasions have been less studied than terrestrial ones, and the ecological and evolutionary consequences of most invasions remain unknown (Ricciardi & MacIsaac 2011).
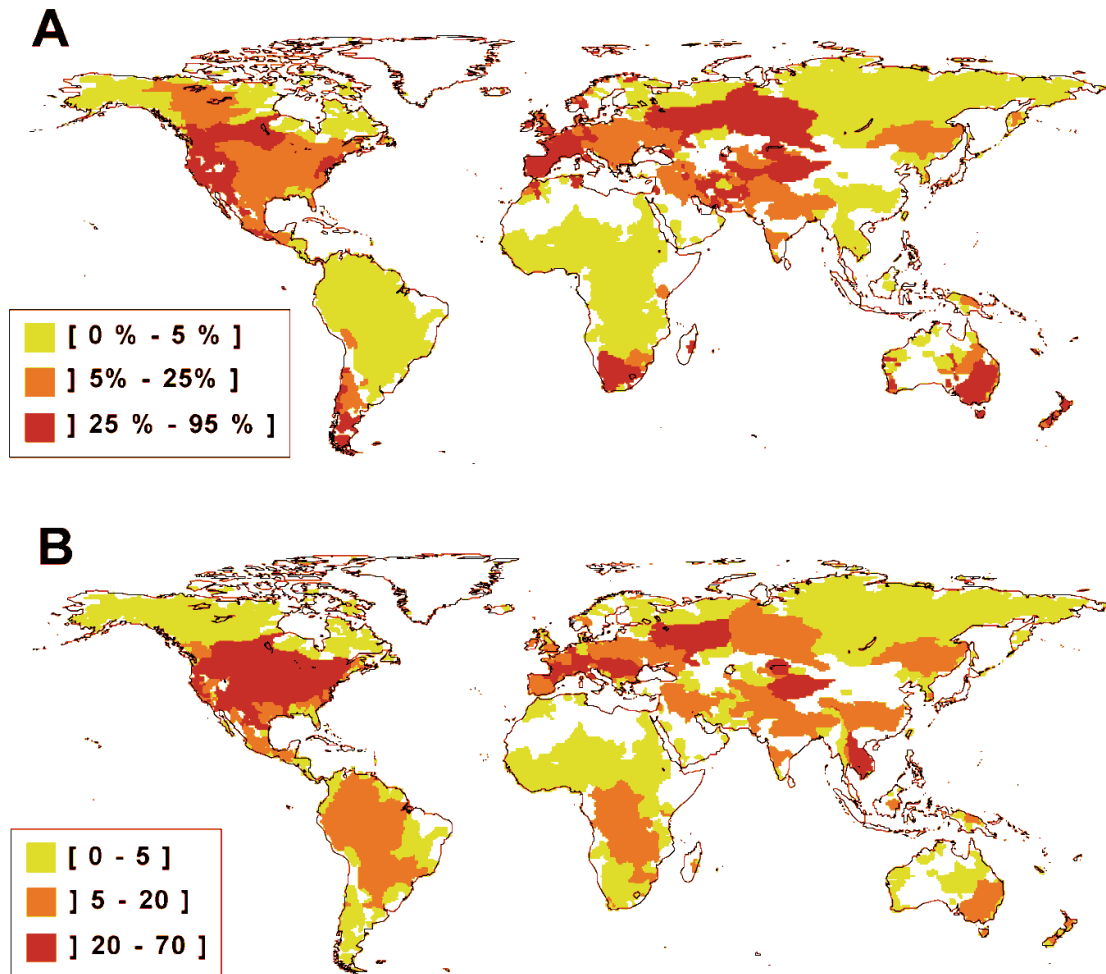


Figure 2: Worldwide distribution of non-native freshwater fish: A) percentage of non-native species per basin (i.e. the percentage of non-native species richness relative to total species richness) and B) non-native species richness per basin (from Leprieur *et al.* 2008).

Many human activities are involved in the introduction of non-native aquatic species to areas beyond their native ranges (Leprieur *et al.* 2008; Ricciardi & MacIsaac 2011). Socioeconomic factors (e.g. human population density, gross domestic product) are known

to be important predictors of exotic species richness across regions (Dalmazzone 2000; García-Berthou *et al.* 2005; Westphal *et al.* 2008; McGeoch *et al.* 2010), and environmental (e.g. temperature) and latitudinal factors influence their success (Cassey *et al.* 2005). Thus the identification of introduction routes and mediating factors are important for genetics, phylogeography and for preventing new invasions (Ricciardi & Rasmussen 1998; García-Berthou *et al.* 2005; Clavero & García-Berthou 2006; Schlaepfer *et al.* 2011). Understanding the ecosystem impacts of non-native species at macro-ecological scales is useful for conservation purposes (Marr *et al.* 2013).

*Mosquitofishes as invasive species*

*Gambusia holbrooki* Girard, 1859 and *Gambusia affinis* (Baird & Girard, 1853) are freshwater poeciliid fishes native to part of the United States of America and Mexico (Fig. 3), commonly named "Eastern Mosquitofish" and "Western Mosquitofish", respectively (Meffe & Snelson Jr. 1989; Rauchenberger 1989; Pyke 2008).



Figure 3: Native geographic distributions of *Gambusia holbrooki* (left) and *Gambusia affinis* (right) (from U.S. Geological Survey 2015).

They are generally omnivorous, feeding on insects, crustaceans and many other taxa (Clem & Whitaker Jr. 1995; García-Berthou 1999; Gkenas *et al.* 2012). They are characterized by fast growth, high reproductive potential, short gestation period (22–25 days, but can extend from 15 to 50 days depending on water temperature, season, and locality), aggressive behaviour and capacity to adapt their life history to particular environments (Krumholz 1948; Pyke 2008). Females show continuous (indeterminate) growth, while males have more or less determinate growth, i.e. they stop growing (or grow very little) when they reach sexual maturity (Fig. 4) (Hughes 1986); the largest total length can be 57 mm for female and 34 mm for male (Krumholz 1948). Particularly, females can

store viable male sperm for several months after their last mating and may produce multiple broods. Males are generally less than females due to the females, although heavily gravid, are more resistance than are the males, and the mature males, having a greater mortality rate than the females, are less numerous in long standing populations with the result that unequal sex ration occur in adult stocks (Krumholz 1948).

   *G. affinis* and *G. holbrooki* are very similar in morphology (Fig. 4) and ecology, and were regarded as three separate species (with *G. patruelis*, which is now considered a synonym of *G. affinis*) around the 1920s, later as two subspecies (*G. affinis affinis* and *G. affinis holbrooki*) of a single species around the 1950s, and finally distinguished again as two separate species after studies on genetics (Wooten *et al.* 1988) and gonopodia morphology (Fig. 5) (Rauchenberger 1989). Walters & Freeman (2000) also provide differences in the number of fin rays to help separate these two species (see Table 1).



Figure 4: *Gambusia holbrooki* from the Ter Vell lagoon (L'Estartit, northeastern Spain) (left) and *Gambusia affinis* (right, photo by U.S. Geological Survey 2015): the individuals are males (bottom) and females (top).
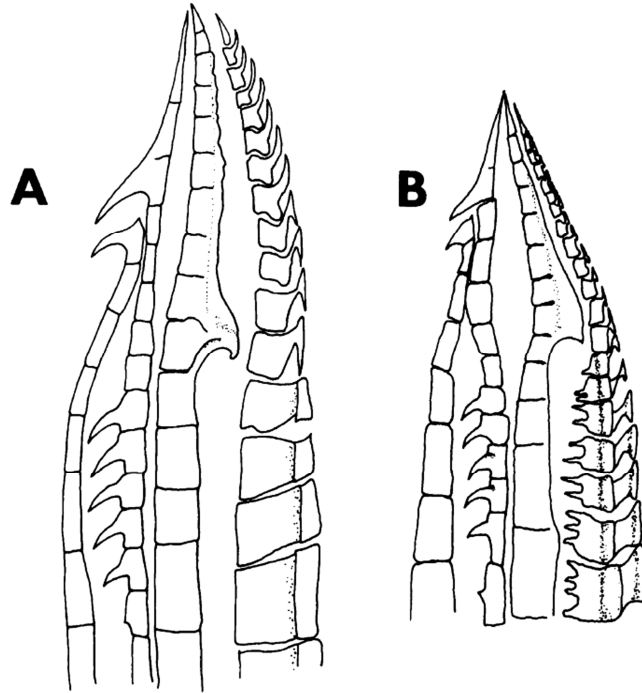
Figure 5: Gonopodia of A) *Gambusia affinis* and B) *G. holbrooki* (from Rauchenberger 1989).

Table 1: Review of fin ray counts in *Gambusia affinis* and *Gambusia holbrooki* (from Walters & Freeman 2000).

| Citation | G. affinis | | G. holbrooki | |
|---|---|---|---|---|
| | Dorsal | Anal | Dorsal | Anal |
| Baird and Girard (1852) | 6 | 8 | — | — |
| Girard (1859) | — | — | 8 | 9 |
| Regan (1913) | 6, 7 | 10, 11 | 8 | 10 |
| Hubbs (1926) | 7, 8 | — | (6) 7 (8) | 9, 10 |
| Hubbs and Lagler (1964) | 6 | 9 | 7 | 10 |
| Lydeard et. al (1991)[a] | 6 | 9 | 7 | 10 |
| Page and Burr (1991) | 6 | — | 7 | — |
| Etnier and Starnes (1993) | 7 | 10 | 8 | 10 |
| Jenkins and Burkhead (1994) | — | — | 7 | 8 (9) |
| Angus and Howell (1996)[a] | 6 | 9 | 7 | 10 |
| Mettee et al. (1996) | 6 | 9 | 8 | 10 |
| Walters and Freeman (this paper)[b] | (6) 7 | 10 | 8 | 11 (12) |

[a] Used counts provided by Hubbs and Lagler (1964).
[b] Following the methods of Rivas (1963).

For the above reasons, there is a widespread confusion in the records and history of introductions of both species including in the most widely used databases and scientific papers. *G. affinis* and *G. holbrooki* are often cited wrongly or together; especially when *G. affinis* is cited, it is often unclear the species involved (Vidal *et al.* 2010). For instance, strong disagreements on the worldwide distribution of *G. affinis* between two widely used

databases (Global Biodiversity Information Facility (GBIF) and FishBase, last consulted in January 2015) can be observed by comparing Figures 6 and 7).
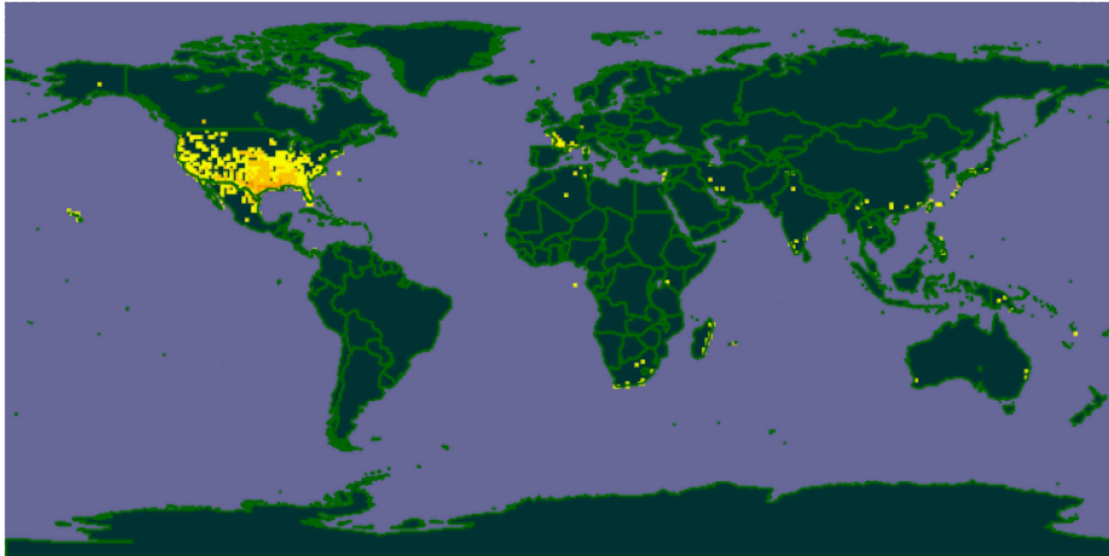


Figure 6: Geographical distribution of *Gambusia affinis* according to GBIF (2013).
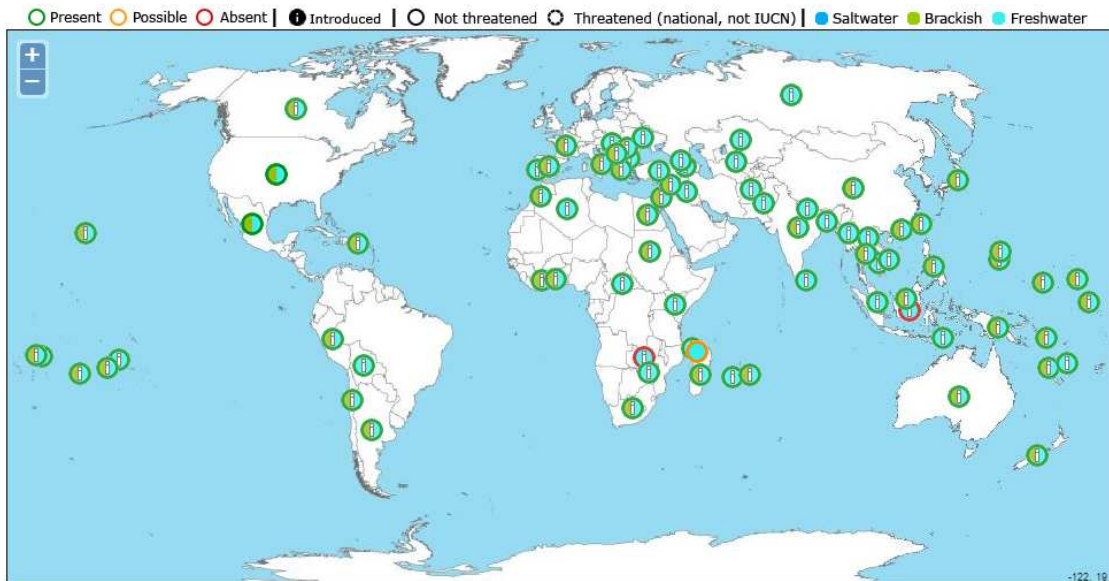


Figure 7: Geographical distribution of *Gambusia affinis* according to FishBase (Froese & Pauly 2014, retrieved in January 2015).

These two mosquitofish species have been introduced worldwide since the early 1900s, as  a biological control to lower mosquito populations (Gerberich 1946; Krumholz 1948; Pyke 2005). In Europe, *G. holbrooki* were first introduced to Spain from the USA in 1921, then transferred to Italy in 1922, and from Italy they were transferred to many other

countries (Vidal *et al.* 2010). In December 1927, *G. affinis* (referred to as *G. patruelis*) were apparently brought from Carbondale (Illinois) to ponds in Rovigno and Valle d'Istria, Italy because it was thought to resist cold weather better than *G. holbrooki* (Vidal *et al.* 2010). However, only *G. holbrooki* has been recently recorded throughout the European continent (Vidal *et al.* 2010).

*Ecological impacts of mosquitofishes*

These two mosquitofish species are among 100 of the world's worst invasive alien species (Lowe *et al.* 2000), have established in all continents except Antarctica, and have been implicated in the local extinction and decline of numerous endemic and native species (Welcomme 1988; García-Berthou *et al.* 2005; Pyke 2008). The establishment rate of *G. holbrooki* was the highest among the top ten most introduced fish species (Table 2) (García-Berthou *et al.* 2005).

Table 2: Invasion transitions of the 10 most frequently introduced aquatic species in the world plus 2 additional species (obtained from the Food and Agriculture Organization's Database on Introductions of Aquatic Species) (from García-Berthou *et al.* 2005).

| | Number of introductions | Percentage established | $P$ | Percentage causing ecological effects | $P$ |
|---|---|---|---|---|---|
| *Oreochromis mossambicus* | 172 | 85.9 | <0.0005 | 81 | 0.26 |
| *Cyprinus carpio* | 124 | 82.0 | 0.91 | 86 | 0.47 |
| *Oncorhynchus mykiss* | 99 | 53.8 | 0.38 | 88 | 0.51 |
| *Ctenopharyngodon idella* | 91 | 11.3 | 0.06 | 60 | 0.71 |
| *Hypophthalmichthys molitrix* | 79 | 26.8 | 0.20 | 75 | 0.24 |
| *Oreochromis niloticus* | 78 | 70.2 | <0.0005 | 75 | 0.25 |
| *Gambusia* spp. | 67 | 96.8 | 0.43 | 50 | 0.15 |
| *Micropterus salmoides* | 64 | 72.9 | 0.78 | 86 | 0.19 |
| *Aristichthys nobilis* | 55 | 19.6 | 0.27 | 80 | 0.48 |
| *Carassius auratus* | 54 | 92.3 | 0.63 | 75 | 0.37 |
| *Lepomis gibbosus* | 25 | 91.3 | 0.01 | 71 | — |
| *Procambarus clarkii* | 24 | 88.9 | 0.62 | 86 | 0.74 |

**Note:** The number of introductions refers to introductions to different countries. Several introductions of the same species into the same country are not considered. The *P* values correspond to *G* tests of independence of establishment or presence of ecological effects (yes/no) in different continents.

They are known to reduce population density of zooplankton and benthic macroinvertebrates, and sometimes increase level of chlorophyll, suspended solids, nutrients and phytoplankton density in the water column (Hurlbert *et al.* 1972; Margaritora

*et al.* 2001), thus having top-down ecosystemic effects. Negative impacts of mosquitofish on ecosystem features, invertebrates, fish and amphibians have been well demonstrated (Pyke 2008; Stockwell & Henkanaththegedara 2011, see Table3).

In the Iberian Peninsula, mosquitofish has been widely introduced in ponds, wetlands and lagoons since the 1920s (Elvira & Almodóvar 2001), also displaying a strong invasive character throughout this region. Indeed, the main impact on endemic fish fauna is via trophic competition and aggression towards endemic cyprinodontiforms, such as Iberian toothcarp *Aphanius iberus* (Valenciennes 1846) and Valencia toothcarp *Valencia hispanica* (Valenciennes 1846), as shown through experiments (e.g. Rincón *et al.* 2002; Caiola & de Sostoa 2005) and observational field studies (e.g. Alcaraz & García-Berthou 2007).

Table 3: Experimental evidence of negative impacts of mosquitofish (*Gambusia affinis* and *G. holbrooki*) (from Stockwell & Henkanaththegedara 2011).

| Taxa | Impact | Overall impact | Reference |
|---|---|---|---|
| **Impacts on invertebrates** | | | |
| *G. affinis* | Reduced zooplankton and insect populations; high algal densities | Negative | Hurlbert et al. 1972 |
| *G. affinis* | Reduced aquatic macroinvertebrate abundance in rice fields | Negative | Farley & Younce 1977 |
| *G. affinis* | Reduced pelagic aquatic invertebrates; increased algae and some benthic invertebrates | Negative | Hurlbert & Mulla 1981 |
| *G. affinis* | Reduction of aquatic macroinvertebrates in rice fields | Negative | Bence 1988 |
| **Impacts on fish** | | | |
| *G. affinis* | Replacement of *Poeciliopsis occidentalis* by predation | Negative | Meffe 1985b |
| *G. holbrooki* | Reduced population growth of *Heterandria formosa* | Negative | Lydeard & Belk 1993 |
| *G. holbrooki* | Size-selective predation on small *Heterandria formosa* in experimental mesocosms | Negative | Belk & Lydeard 1994 |
| *G. holbrooki* | Reduced growth and lack of egg survival of *Pseudomugil signifer* | Negative | Howe et al. 1997 |
| *G. holbrooki* | Caudal fin damage and mortality of *Edelia vittata* | Negative | Gill et al. 1999 |
| *G. holbrooki* | Heavy predation on *Aphanius iberus* and *Valencia hispanica* juveniles | Negative | Rincon et al. 2002 |
| *G. affinis* | Reduced growth and survival of *Iotichthys phlegethontis* young of year | Negative | Mills et al. 2004 |
| *G. affinis* | Reduced population size and biomass of *Cyprinodon tularosa* | Negative | Rogowski & Stockwell 2006a |
| *G. affinis* | No impact on larval survival; increased body growth of *Siphateles bicolor mohavensis* | Neutral | Henkanaththegedara & Stockwell, unpublished data |
| **Impacts on amphibians** | | | |
| *G. affinis* | Elimination of *Hyla regilla* tadpoles | Negative | Hurlbert & Mulla 1981 |
| *G. affinis* | Predation on *Taricha torosa* larvae | Negative | Gamradt & Kats 1996 |
| *G. affinis* | Predation on *Hyla regilla* tadpoles | Negative | Goodsell & Kats 1999 |
| *G. affinis* | Delayed metamorphosis and reduced growth rates of *Rana aurora draytonii* | Negative | Lawler et al. 1999 |
| *G. holbrooki* | Reduced survival of endangered *Litoria aurea* tadpoles | Negative | Hamer et al. 2002 |

However, there are many environmental factors that influence the ecological success and impact of mosquitofish, such as salinity (Alcaraz *et al*. 2005), latitude (Carmona-Catot *et al*. 2011), temperature (Carmona-Catot *et al*. 2013), and habitat (Patimar *et* al. 2011).

Meta-analysis is a statistical technique that is very useful for synthesizing research findings across studies and ecosystems (Gurevitch & Hedges 1999), and can help to understand the overall impacts of invasive species and the factors that mediate them

(Matsuzaki *et al*. 2009; Vilà *et al*. 2011). Meta-analysis is also a powerful approach to explore heterogeneity among studies and identify patterns across species and geographic regions (Stewart 2010).

*Swimming capacity and metabolism*

Mosquitofish is a so-called "limnophilic" species and water flow is known to affect its invasive success (Meffe 1984; Murphy *et al.* 2015). Measurements of metabolism and swimming capacity allow us to determine the influence of physiological tolerance on invasive species success (Plaut 2001; Budy *et al.* 2013; Glazier 2014). Oxygen consumption measurements are usually measured to estimate metabolic rate (Bell & Terhune 1970; Price *et al.* 2012). Critical swimming speed is a standard measurement to evaluate swimming capabilities of fishes (Plaut 2001).

Multiple autoecological aspects have been assessed for mosquitofish in the Iberian Peninsula, such as salinity tolerance, life-history traits, habitat requirements, parasite interactions, aggressive behaviour or prey selection (García-Berthou 1999; Caiola & de Sostoa 2005; Alcaraz & García-Berthou 2007; Benejam *et al*. 2009; Carmona-Catot *et al*. 2013, 2014). From a conservation perspective, these environmental studies are crucial to evaluate different impacts of this fish species on Iberian fauna and also help to effectively control their populations in this region (e.g. Ruiz-Navarro *et al*. 2013). Besides this, swimming speed and metabolic traits are factors of pivotal relevance for ecological and physiological models (e.g. survival, feeding, mating or predator avoidance), particularly in fish as an integral part of aquatic habitats (Plaut 2001; Killen *et al*., 2007; Huang *et al*. 2013). Similarly to other autoecological aspects mentioned above, data on swimming capacity and metabolism level may help to reveal the potential competition for space in the water-column with a variety of native species (e.g. Ward *et al*. 2003), other than toothcarps (*Aphanius iberus*) in still-waters. As an example in the Iberian Peninsula, mosquitofish were observed to disrupt foraging behaviour of the endemic cyprinid *Squalius alburnoides* in flowing-waters (Almeida & Grossman 2012). Furthermore, this information on mosquitofish swimming ability could be used to identify target areas for invasion under fluctuating hydrological conditions (Murphy *et al*. 2015), which may have implications for water management (e.g. flow regulation and abstraction). In particular, the effects of size and sex on locomotion traits would contribute to a better understanding on how mosquitofish adapt to variable local conditions. However to our knowledge, no data on

swimming capacity and metabolism of invasive mosquitofish are known to exist regarding size-sex interactions, as studies on fish locomotion speed commonly assess either males or females only (e.g. Nicoletto 1991; Kolok & Oris 1995; Plaut 2002; Seebacher *et al.* 2012). Given that different properties of the ecological niche (e.g. salinity, trophic level, thermal ranges) have been already assessed for mosquitofish (see citations above), information is especially useful to understand the 'flow niche' of this species in Iberian fresh waters.

# Objectives

The main general objective of this thesis is to contribute to a comprehensive understanding of the ecological success of two worldwide fish invaders (*G. holbrooki* and *G. affinis*). With this aim, we combined a review, a meta-analysis, and an experimental test to address the specific objectives that follow.

   **In Study 1**, we aimed at clarifying the world distribution of *G. holbrooki* and *G. affinis*, to establish the introduction routes and histories of both species and to find out what are the main factors that influence their establishment. We hypothesized that there would be clear spatial patterns in introduction routes, linked to human activities, and that temperature would strongly affect the probability of establishment.

   **In Study 2**, we aimed at performing a meta-analysis to synthesize the ecological impacts of both *G. holbrooki* and *G. affinis*. Mosquitofish are known to reduce the density of zooplankton and benthic macroinvertebrates, and sometimes increase the levels of chlorophyll, suspended solids, nutrients and phytoplankton density in the water column. We hypothesized that the magnitude of effects would decrease top-down. In addition, based on the literature we hypothesized that mosquitofishes would have clear competitive and predatory effects on native fishes and other taxa but effects would be heterogeneous and context-dependent. For instance, the methods used, such as dimensions of tanks or aquaria or fish sizes, might affect the conclusions of the experiments. We hypothesized that there should be a positive correlation between density and size of mosquitofish and the ecological impact, and negative correlations with dimension of tanks or absolute latitude (lower temperatures).

   **In Study 3**, we aimed at investigating the critical swimming capacity and metabolism of *G. holbrooki* focusing on sex and size effects, to evaluate the influence of water flow on

its invasive success. We compared size and sex variations in swimming speed and associated oxygen consumption (i.e. as an index of metabolism level) for a wild mosquitofish population located in an Mediterranean coastal lagoon. We hypothesized that critical swimming capacity or metabolic rates would increase with fish size and that there would be a significant difference in swimming capacity and metabolism between males and females.

It has been increasingly recognized that the success and impact of invasive species is context- (e.g. Spooner & Vaughen 2006;  Alcaraz *et al*. 2008; Blanchet *et al.* 2009; Pyšek *et al*. 2012) and scale-dependent (e.g. Davies *et al*. 2005; Fridley *et al*. 2007). Ecological interactions are context-dependent when the sign or magnitude of effects is a function of the biotic or abiotic setting (Chamberlain *et al*. 2014). The three studies in this thesis all aim at understanding the context-dependency of the invasive success and impact of mosquitofishes: the first study ask questions at the regional or country level (e.g. how does temperature affects the establishment success of mosquitofishes?), the second and third studies are at the local level (e.g. how does temperature or the local fauna affects the ecological impacts of mosquitofishes?). The three studies are also complementary of each other because the first one benefits from the large temporal and spatial scales, high realism and generality of natural experiments, whereas the other two benefit from the conclusiveness and control of experiments (Diamond 1986; Keddy 2001).

# Methodology

## Study 1: Review of the world distribution and introduction correlates of mosquitofishes

*Clarification of mosquitofishes distribution*

We reviewed the introduction records in the scientific literature and the most widely used databases on invasive species, including the Food and Agriculture Organization's Database on Introduction of Aquatic Species (DIAS), FishBase, the Global Invasive Species Database (GISD), and the Global Biodiversity Information Facility (GBIF) (see Table 5 for source of the databases). DIAS was initiated by a former chief (Robin L. Welcomme) of the FAO in the early 1980s and originally considered only freshwater species. The original raw records were published by Welcomme (1988) and discussed in Welcomme (1991, 1992). FishBase was initiated at the WorldFish Centre in Penang, Malaysia in 1990, in collaboration with the FAO and many other partners, and the first version appeared in a CD version in 1995 and first released on the internet in 1998 (Froese & Pauly 2012); their introduction records are in part based on the DIAS database published in 1988 but have subsequently been updated with independent records (FAO 2012). GISD is an online database focusing on invasive alien species that threaten native biodiversity, which was developed by the Invasive Species Specialist Group of the World Conservation Union–IUCN's Species Survival Commission (GISD 2012). GBIF is an international organization that was established in 2001 to encourage the creation of the world's primary database on biodiversity accessible via internet (GBIF 2012).

We obtained all introduction records of *G. holbrooki* and *G. affinis* from FishBase on 20 October 2012, including the countries of origin and destination, the date of introduction, whether the species has established in the wild and the data sources as a first database. We extensively searched the literature and internet databases for published records of *G. holbrooki* and *G. affinis* introduced to the wild. We revised the original FishBase database with the following criteria: i) we noted new records of *G. holbrooki* and *G. affinis* introduced to the wild (and whether they have established or not, if known) to a given country; we did not consider references of mosquitofish used in captivity (experiments) but not recorded in the wild; ii) because *G. holbrooki* and *G. affinis* were distinguished again as different species (not subspecies) only after the paper by Wooten *et al.* (1988) and older

and recent records often mixed the two species, we noted papers that explicitly stated that they used morphology (e.g. Rauchenberger 1989 for gonopodia; Walters & Freeman 2000 for fin rays) or genetics to identify the species; iv) we assumed that all introductions to Europe before 1927 were of *G. holbrooki*, because in Europe *G. holbrooki* was first introduced to Spain from the USA in 1921, then transferred to Italy in 1922, and from Italy transferred to many other countries (Vidal *et al.* 2010). *G. affinis* (referred to as *G. patruelis*) were apparently brought from Carbondale (Illinois) to ponds in Rovigno and Valle d'Istria, Italy in December 1927 (Vidal *et al.* 2010); (*v*) we similarly revised the species involved based on recent citations and the country of origin of the introduction; and (*vi*) we marked with a question symbol for the records in the four databases cited only one species, which seems clearly established in a specific country, but no recent literature is available to clarify the species involved (hereafter, "*G. holbrooki*?" or "*G. affinis*?").

We used FishBase because it was the most comprehensive geographical database on these two fish species, with 109 records (29 *G. holbrooki* and 80 *G. affinis*). From the other databases, DIAS contains 96 records (24 *G. holbrooki* and 72 *G. affinis*), and most of those were available in FishBase. In GISD, 27 out of 88 records (21 *G. holbrooki* and 67 *G. affinis*) were obtained from FishBase, and in GBIF, 38 out of 91 records (34 *G. holbrooki* and 57 *G. affinis*) were also obtained from FishBase. The data collected were compared among the four different databases. For all statistical analyses, we replaced ranges for the year of introduction with the midpoint and assumed the date of the first published record as the date of first introduction for unknown dates. We noted more than one introduction record per country but we considered only the first introduction in the statistical analyses.

*Socioeconomic variables and introduction correlates*

As potential predictors of introduction history, we compiled for each country: the total area, human population density (PopDensity), average gross domestic product per capita (GDPC), export as percentage of gross domestic product (Exports), per cent area of forest coverage (Forests) and per cent area of agricultural land (AgriLand) in 2000 (all obtained from Gapminder 2012; see the website for original sources of the data); latitude and longitude (Erle *et al.* 2008); and daily minimum and mean temperature, precipitation and frost day frequency for 30 years (1961–1990) from the Tyndall Centre (Mitchell *et al.* 2002, 2004). We selected these socioeconomic factors because they are known to be significant predictors of introductions and richness of alien species (Vilà & Pujadas 2001;

García-Berthou *et al.* 2005; Hulme & Weser 2011) and the climatic factors because they influence mortality and establishment success of mosquitofish (Maglio & Rosen 1969; Cherry *et al.* 1976; Haynes 1993; Pyke 2008). We also compiled socioeconomic data from the 1960s, but these were not used in the analyses because they were highly correlated with recent descriptors and often not available for many countries. We used all these predictors to find out what are the best predictors for a number of response variables for each species: the probability of introduction to the country (hereafter, RECEIVING COUNTRY), establishment success (ESTABLISHMENT), number of donations (number of times that the species has been introduced from the different countries) to other countries (ORIGINATING COUNTRY), and the date of first introduction (INTRODUCTION DATE).

*Statistical analyses*

We used the 'rworldmap' package (South 2011) in the R statistical software (R Core Team 2014), to display a world map of the current distributions of *G. holbrooki* and *G. affinis*, and the 'rworldmap' with 'geosphere' packages (Hijmans *et al*. 2012) to draw world maps of introduction routes. Log-transformation was applied to several variables to satisfy the assumptions (e.g. normality, homoscedasticity, and linearity) of many of the statistical procedures: $\log_{10} x$ for GDPC (lg_GDPC), daily precipitation (lg_Precip) and country area (lg_Area); $\log_{10}(x - a + 1)$ for variables such as daily minimum temperature (lg_MinTemp) and daily mean temperature (lg_MeanTemp) that take negative values (*a* was the minimum value of the variable); and $\log_{10}(x + 1)$ for frost day frequency (lg_FrostDays) and donation numbers of *G. holbrooki* or *G. affinis* to other countries. We used generalized additive models (GAMs) with Poisson errors and the 'gam' function in the 'mgcv' R package (Wood 2006) to fit the smooth curves of the relationships between number of donations to other countries and date of first introduction to the country for the two *Gambusia* species. Stepwise selection procedures were used to obtain the best model, according to Akaike's information criterion (AIC). The Student's *t*-test was used to compare mean difference of donation numbers or date of first introduction between *G. holbrooki* and *G. affinis*; and Levene's test was also carried out to test for differences in variance. Generalized linear models with binomial errors and logit link functions were used to relate establishment success (yes/no) and date of first introduction for each species.

To compare the distances of introductions for the two species, we estimated the distances between the countries of origin and destination with the 'geosphere' package in

R, based on the country geographical coordinates. To test for the preferential directions for each species, we used the Rayleigh test for circular uniformity (Jammalamadaka *et al*. 2001). To test whether the mean direction of dispersion differed between the two species, we used the Watson's two-sample test of homogeneity (Jammalamadaka *et al*. 2001). We also fitted a circular-linear regression model (Jammalamadaka *et al*. 2001) for a circular dependent variable (i.e. distribution direction) and a linear independent variable (i.e. year of introduction) to test whether the direction changed with year of introduction for each species. We performed all circular statistics with the 'circular' package (Agostinelli & Lund 2013) in R.

We used the 'party' package (Hothorn *et al*. 2006) in R to estimate what are the most important predictors mediating the RECEIVING COUNTRY, ORIGINATING COUNTRY, ESTABLISHMENT, and INTRODUCTION DATE of the two species. We used the 'cforest' function with the default options and "mtry" (number of randomly preselected predictor variables) = 4, following square root of the number of predictor variables as a suggested by Strobl *et al*. (2009b). The absolute values of the lowest ranking predictor were added to the plots as indicative of informative and important predictors (Strobl *et al.* 2009b). We used the 'party' rather than the more widely used 'randomForest' R package (Liaw & Wiener 2002) to avoid the biased variable selection and variable importance for predictor variables when they are of different types (e.g. scales, categories) or in the case of correlated predictors (Strobl *et al.* 2007, 2008, 2009a).

**Study 2: Meta-analysis of the ecological impacts of mosquitofishes**

*Literature search*

We conducted a literature search to gather quantitative evidence from experimental and observational studies on the ecological impacts of invasive mosquitofishes, *G. affinis* and *G. holbrooki*. Searches were conducted on 23 January, 2014 in the Web of Knowledge (https://webofknowledge.com), using keyword combinations (*Gambusia* or mosquitofish* and impact* or effect* or ecosystem* or ecolog* or native* or competi*) in the topic for all publication years. We then screened the retrieved references from the database to meet the criteria of the meta-analysis. A study had to meet the following two criteria to be included in the analysis: i) it evaluated the effects of mosquitofish on ecosystem features (e.g. chl-*a* concentration, nutrient concentrations, suspended solids, etc.) or biota (e.g. density of zooplankton or benthic invertebrates, fish survival, growth or fecundity) through either

manipulative experiments or observational studies (before–after invasions); and ii) the study had a control group with no mosquitofish or native species alone. When a response variable was measured at different times (e.g. sampling at different dates or repeated-measure experimental designs), we only used the final measurement, but when the same article examined different treatments (e.g. fish size, fish density, temperature, etc.), we considered each of these separately. These criteria discarded studies that examined behavioural interactions but had no control group (with no mosquitofish). We also did not include studies using only mosquitofish cues but not real presence and studies consisting of more complex treatments (e.g. common carp + mosquitofish vs. control).

*Data extraction*

A total of 62 articles (see Appendix for list of articles) representing 577 cases (501 for biota and 76 for ecosystem features, 346 for *G. holbrooki* and 231 for *G. affinis*) fit our inclusion criteria (see Fig. 8 for geographical distribution of publications).



Figure 8: Geographical distribution of publications used in the meta-analysis.

We compiled all response variables reported to describe the effects of mosquitofish (e.g. chlorophyll and nutrient concentrations, zooplankton density, fish abundance, number of aggressions, etc.) (Table 4) and the following potential explanatory variables: experiment vs. observational study; volume and depth of the experimental setting; experimental duration, latitude, water temperatures; and density of the mosquitofish or target species used when reported. Where latitude was not reported in a study, it was estimated to the nearest decimal degree from maps using the site descriptions. We recorded the mean, dispersion measure reported (SD, standard deviation; SE, standard error; or CI, confidence interval) and sample size for each response variable reported and each treatment. Means

and dispersion statistics from published figures were obtained using the DATATHIEF III software (Thumers 2006), when the exact values were not reported in the articles. The SE or CI obtained were converted into SD; for those cases where only mean values were reported and not variability measures (30 cases), we estimated the standard deviation ($\widetilde{SD}$) of a given study (denoted by $j$) by using available data from other studies in the dataset following Lajeunesse (2013), calculated as:

$$\widetilde{SD}_j = \overline{X}_j \left( \frac{\sum_i^k SD_i}{\sum_i^k \overline{X}_i} \right)$$

where $\overline{X}_j$ is the observed mean of the study with missing information, $i$ denotes complete information and $k$ is the number of $j$th studies in the dataset.

*Meta-analysis*

To compare the ecological impacts of mosquitofish between treatment (or after-invasion) and control (or before-invasion) groups, we calculated Hedges' $d$ as a measure of effect size (Hedges & Olkin 1985). Hedges' $d$ is an estimate of the standardized mean difference that is not biased by small sample sizes (Rosenberg *et al.* 2000) and is a commonly used measure of effect size in ecological studies (Møller & Jennions 2002). Following Rosenberg *et al.* (2000), we calculated $d$ as:

$$d = \frac{(\overline{X}_T - \overline{X}_C)}{S} J$$

where $\overline{X}_T$ is the mean of the treatment group, $\overline{X}_C$ is the mean of the control group, and $S$ is the pooled standard deviation, calculated as:

$$S = \sqrt{\frac{(N_T - 1)S_T^2 + (N_C - 1)S_C^2}{N_T + N_C}}$$

where $S_T, S_C$ are the standard deviations for treatment and control groups, respectively. $J$ is a weighting factor based on the number of replicates for the treatment ($N_T$) and control ($N_C$) groups. $J$ was calculated as:

$$J = 1 - \frac{3}{4(N_C + N_T - 2) - 1}$$

The variance of Hedges' $d$ ($V_d$) was calculated as:

$$V_d = \frac{N_C + N_T}{N_C N_T} + \frac{d^2}{2(N_C + N_T)}$$

Hedges' $d$ is a unit-free index which ranges from $-\infty$ to $+\infty$; negative values of $d$ denote negative effects of mosquitofish on the measured response variable between treatment and control groups (no mosquitofish or native fish species), and vice versa. Hedges' $d$ calculations and statistical analysis were conducted with 'metafor' package (Viechtbauer 2010) in R statistical software (R Core Team 2014). We changed the sign of the effect sizes for those response variables in which a low value indicates the same as a high value for related variables (e.g. decreased mortality is equivalent to increased survival; see Table 4 for details).

We used random-effects models with default restricted maximum-likelihood (REML) method, because it is an approximately unbiased and quite efficient estimator (Viechtbauer 2005), to test whether mean effect sizes of each group or variable type of biota and ecosystem features differed significantly from zero. We classified the biota as fish, amphibians, macroinvertebrates, macrophytes, zooplankton, phytoplankton, picoplankton, periphyton, and bacteria. For variable types, we grouped them into density/biomass (e.g. population growth), size/stage (e.g. length, development stage), life history, diversity, feeding behaviour, agonistic behaviour, other behaviour (microhabitat use). For ecosystem features, we grouped them into concentration of chemicals, transparency, and other physical and chemical properties (see Table 4 for further details). For the nine biota groups, we grouped them by family for fish and by order or higher taxonomic levels for other biota.

Table 4: Classification of response variables by type and taxonomic group used for the meta-analysis.

| Taxonomic group | Variable type | Response variables |
|---|---|---|
| Fish | | |
| | Density/biomass | Survival, population growth, biomass, total weight harvested, survival rate, and mortality (–) |
| | Size/stage | TL, and SL |
| | Life history | Offspring number, specific growth rate, instantaneous growth rate, and gonadosomatic index |
| | Feeding behaviour | Prey captured, foraging efficiency, foraging success, prey selection indices, total food fed, and food conversion ratio |
| | Agonistic behaviour | Aggressive acts received (–), aggressive acts performed, bites received (–), chase numbers received, orientations performed, fin damage index (–), and caudal fin loss (–) |
| | Other behaviour | Distance from floating cover, distance from submerged cover, distance from the feed station, in "predator" half, swimming, and on bottom |
| Amphibians | | |
| Anura | Density/biomass | Mass, survival or mortality (–) |
| | Size/stage | Snout-vent length, development stage, final mass of hatchings, and metamorphosing rate |
| | Life history | Mass at metamorphosis, metamorphic numbers produced, metamorphosing period (–), time to hatching (–), and proportion of eggs hatching. |
| | Diversity | Species richness |
| | Agonistic behaviour | Number of tadpoles injured (–) |
| | Other behaviour | Time active, proportion active, and proportion hiding (–) |
| Urodela | Density/biomass | Mass, and survival rate |
| | Life history | tail-body ratio |
| | Agonistic behaviour | Gill damage (–), and leg damage (–) |
| Macroinvertebrates | | |
| Diptera | Density/biomass | Abundance, and density |
| | Diversity | Number of taxa |
| | Feeding behaviour | Larvae consumption (–) |
| Other insects | Density/biomass | Abundance, and density |
| Other crustaceans | Density/biomass | Abundance, and survival |
| | Diversity | Simpson's diversity index |

| | | |
|---|---|---|
| Mollusca | Density/biomass | Abundance, and density |
| Other invertebrates | Density/biomass | Density, and abundance |
| | Life history | Linear dimension |
| Macrophytes | Density/biomass | Biomass, and coverage |
| Zooplankton | | |
| Cladocera | Density/biomass | Density, biomass, and abundance |
| | Size/stage | Body length |
| | Life history | Offspring number, brood number, clutch size, body size at maturity, and age at first reproduction |
| Copepod | Density/biomass | Abundance, and density |
| Copepoda Calanoida | Density/biomass | Abundance, and density |
| Copepod Cyclopoida | Density/biomass | Density, and mass |
| | Size/stage | Linear dimension |
| Ostracoda | Density/biomass | Mass, and density |
| | Size/stage | Linear dimension |
| Rotifera | Density/biomass | Abundance, density, and mass |
| | Diversity | Species richness, and Simpson's diversity index |
| Other zooplankton | Density/biomass | Abundance, mass, and density |
| | Size/stage | Microcrustacean abundance |
| | | Abundance, density, and biovolume |
| | Diversity | Microcrustacean richness |
| Phytoplankton | Density/biomass | Abundance, density, biovolume, biomass, and phytoplankton fluorescence |
| | Diversity | Genera richness |
| Picoplankton | Density/biomass | Density, and biovolume |
| Periphyton | Density/biomass | Biomass, biovolume, density, and abundance |
| Bacteria | Density/biomass | Density, and biovolume |
| Ecosystem features | Chemicals | Ammonia, nitrate, $NO_x$, total nitrogen, orthophosphate, soluble reactive phosphorus, total phosphorus, N/P, dissolved iron, dissolved manganese, and dissolved organic carbon |
| | Transparency | Chl-*a* (–), SS (–), Secchi depth, and turbidity (–) |
| | Other physical and chemical properties | Conductivity, DO, and pH |

Note: "–" means the sign of the effect size of the response variables was changed; because of opposite meaning of some variables (e.g. increased mortality in presence of mosquitofish is equivalent to decreased survival).

Because the effect sizes of observational and experimental studies ($t = 0.290$, d.f. = 1.12, $P = 0.816$) and of *G. holbrooki* and *G. affinis* ($t = -1.848$, d.f. = 522.46, $P = 0.065$) were not significantly different and the limited sample size, we ignored these sources of variation and pooled the data to analyse other apparently more important predictors of

effect sizes (e.g. taxonomic group and variable types). We tested whether mean effect sizes for each group were significantly different from zero and performed forest plots of the results (Jennions *et al.* 2013; Lajeunesse 2013). Forest plots are graphical overviews of the results to illustrate individual point estimates and their CIs (Lewis & Clarke 2001). When a CI does not include zero, it indicates a statistically significant effect size (Gurevitch *et al.* 1992). Total heterogeneity ($Q_T$) was tested with Cochran's $Q$-test (Cochran 1954), and the percentage of total variation across studies due to heterogeneity ($I^2$ statistic) (Higgins & Thompson 2002) was also obtained to summarize the importance of the heterogeneity (<25%, low heterogeneity; 25–50%, moderate; 50–75%, high; 75–100%, very high) within study groups (Higgins *et al.* 2003). The $Q$ and $I^2$ statistics are commonly used to test for heterogeneity between-study variation (Rosenberg 2013), and the $I^2$ statistic is a better measure of heterogeneity, because it is independent of the number of studies and effect metrics, i.e. type of variable (Higgins & Thompson 2002).

We used mixed-effects models (i.e. meta-regression) with categorical moderator variables, to test whether mean effect sizes differed between groups (e.g. biota or taxonomic groups, variable types), and with continuous predictors to test whether variance in effect sizes covaried with continuous predictors (e.g. water temperature, experimental duration, density of the mosquitofish and target species used; depth and volume of the experimental setting). Absolute latitude was used as a surrogate of water temperatures only for macroinvertebrates and zooplankton (only studies performed outdoors). Other variables compiled (i.e. salinity, dissolved oxygen, air temperatures; sex ratio, mean size of the mosquitofish or target species used) were not used in the analyses because of many missing values. We considered all possible combinations of the continuous predictors and selected the model with the smallest Akaike's information criterion value (corrected for small sample sizes, AICc) (Burnham *et al.* 2011). We tested the models selected with permutation tests (default options), as a preferable alternative (Viechtbauer 2010) to the standard (Wald and likelihood ratio) tests, which assume normality of the observed effects (as well as the true effects in random/mixed-effects models) and rely on the asymptotic behaviour of the test statistics (Follmann & Proschan 1999; Higgins & Thompson 2004).

We tested the model sum of squares ($Q_M$), to explain the amount of heterogeneity of the regression model, and residual sum of square ($Q_E$), to explain the amount of the heterogeneity which is left unexplained after the model is taken into account. A significant

$Q_M$ indicates that mean effect sizes vary significantly among groups for models with only categorical variables and that at least one of the regression coefficients is different from zero for models with continuous predictors; a significant $Q_E$ indicates that there is additional variance in the effect sizes to be explained (Rosenberg 2013).

To address publication bias, we tested our dataset using several methods available in the 'metafor' package. We used the regression test to test for funnel plot asymmetry (Egger *et al*. 1997) for the overall dataset, biota alone and ecosystem features. We estimated fail-safe numbers following Rosenthal (1979), to test whether the number of additional non-significant studies in the analysis would change the results of meta-analysis from significant to non-significant. We also used trim and fill method (Duval & Tweedie 2000) to estimate the number of missing studies on one side of the funnel plot for the overall dataset.

**Study 3: Experiment on the swimming capacity and metabolism of eastern mosquitofish**

*Experimental fish*

Mosquitofish were collected from the Ter Vell lagoon (L'Estartit, northeastern Spain), a small (0.8 ha) and shallow (<1 m deep) water body. This lagoon is located next to (<3 km) the mouth of the Ter River (42º02'44'' N – 3º11'41'' E), which drains into the Mediterranean Sea. More detailed information on the limnology of this lagoon can be found in Badosa (2006). Individuals of the remaining mosquitofishfish were kept in a tank with supplied oxygen (two Aera aerators, portable battery pump) until fully recovery before being released back into the site where captured. Fish collection was carried out during late July 2014. The sampling year, 2014, was a hydrologically average period in the study area (Ministry of Environment Spain 2014). This avoids the effects of particular dry or wet years on the study mosquitofish population and allows the data to be considered as representative for this species in the Mediterranean region of the Iberian Peninsula, increasing the potential generality of the present findings. Also, the study mosquitofish population was sampled from a lentic environment (i.e. a lagoon) for a better assessment of the potential capacity of this species to adapt to the variation in flow conditions.

A sample of mosquitofish (30 females and 30 males) was captured during daylight hours using dip nets (1.5 m long pole, 60 cm diameter net, 1 mm mesh size), transported to

the laboratory within two hours and kept in an outside tank (1,500 litres). Mosquitofish were allowed to acclimatize to the same environmental conditions (e.g. water quality, aquatic vegetation, prey abundance) in the outside tank until the experiment was conducted (≈30 days). The mosquitofish were moved inside laboratory, at least seven days before the trials, fish were separated by sex and allowed to acclimatize to laboratory conditions. In particular, mosquitofish were placed in 90 L stock aquaria ($60 \times 25 \times 75$ cm), containing gravel substrate, conditioned water (conductivity $\approx 389$ µS cm$^{-1}$; pH $\approx 6.59$) and filtered air supply (Tetratec® APS 400, Germany). Moreover, temperature was maintained at $25 \pm 1$ °C under a constant photoperiod (15/09 h light/dark cycle) using 15-W fluorescent lights. Fish were fed to satiation once per day with defrosted frozen bloodworms (*Chironomus* spp.). To avoid post-prandial effects, fish were not fed for 24 h prior to the experiment (Fu *et al.* 2009). Mosquitofish were measured (total length, TL) to the nearest 1 mm and weighed (wet weight, WW) to the nearest 0.1 mg. Mosquitofish is a dimorphic species, with females being larger than males. Specifically in this study, females ranged between 15–44 mm TL and 40.3–650.0 mg WW, and males ranged between 22–35 mm TL and 78.7–374.5 mg WW.

Field sampling and laboratory procedures were adapted from Alcaraz & García-Berthou (2007) and Alcaraz *et al.* (2008), and they complied with all animal use and care regulations of Europe and Spain (specific Licenses were granted for Scientific Research in the administrative region of Catalonia). Fish were collected by trained personnel (i.e. the holder of the Licenses, E. García-Berthou). Thus, no adverse effects were caused to the wildlife in the surveyed lagoon and all non-target fish recovered fully from the netting.

*Swimming performance*

Absolute critical swimming speed ($U_{crit}$, cm s$^{-1}$) was measured using a mini swim tunnel (Fig. 9), of modified Blažka-type design, with a non-turbulent laminar flow and equipped with a continuous-flow respirometer (Loligo® Systems, Denmark). The test section (170 mL volume, 100 mm length $\times$ 26.4 mm internal diameter) was laterally covered with non-reflecting white screens to avoid disturbing fish by 'mirror effects'. The swim tunnel was thermo-regulated with a heater (Eheim Jäger Model 3613, Germany) and supplied from a buffer tank of conditioned water (see particular values above). Individual fish were placed into the respirometer and allowed to acclimatize to an initial velocity of 1.0 cm s$^{-1}$ (i.e. ca. 0.5 body length s$^{-1}$) for 1 h, after which velocity was slowly (within 2-min periods)

increased by 2.0 cm s$^{-1}$ (ca. 1 body length s$^{-1}$) every 20 min, until the fish could no longer swim (i.e. fatigue) (Hammer 1995; Jain *et al.* 1997; Seebacher *et al.* 2012). Fatigue is defined as the point at which fish cannot longer maintain position against the current velocity in the respirometer (Kolok 1991; Plaut 2001).
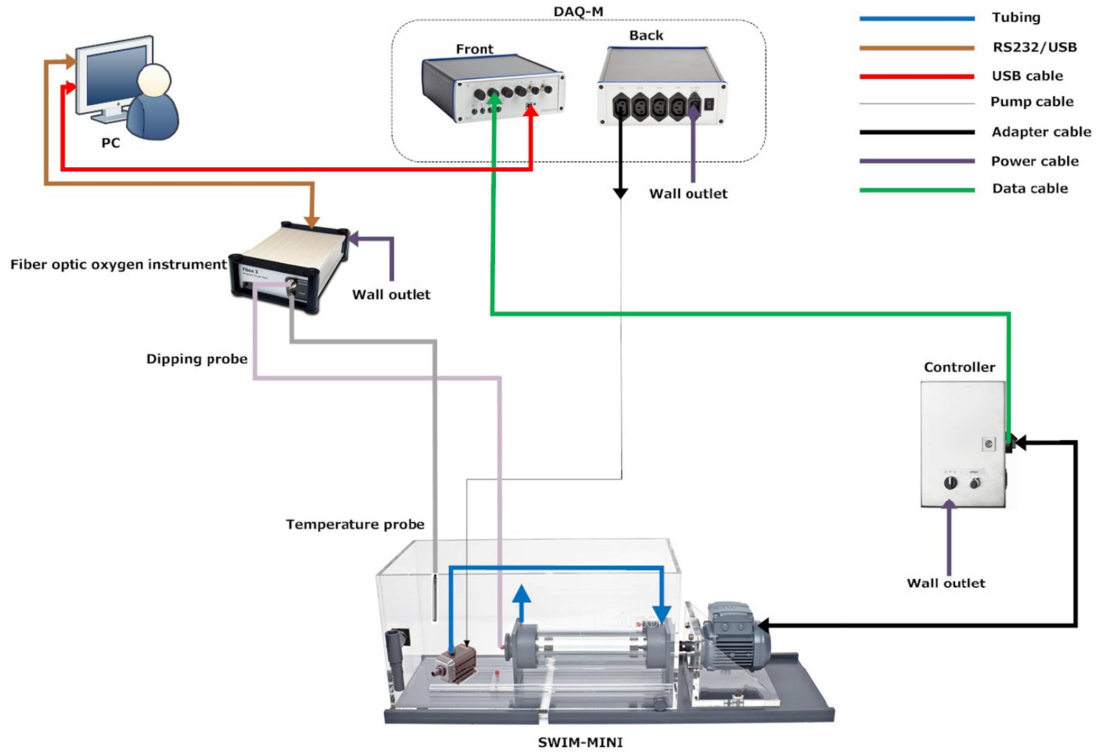


Figure 9: Set-up of the mini-swim tunnel experiment with the fibre optic instrument and sensor (from Loligo® System; http://www.loligosystems.com).

The $U_{crit}$ was calculated as:

$$U_{crit} = U_f + U_i \left(\frac{T_f}{T_i}\right)$$

where $U_f$ is the highest velocity maintained for a full 20 min period, $T_f$ is the time swum at the last velocity increment (min), $T_i$ is the interval time (20 min in this case) and $U_i$ is the velocity increment (i.e. 2.0 cm s$^{-1}$ in this case) (Brett 1964). $U_{crit}$ was not corrected for the 'solid blockage effect' of fish (i.e. the increase of velocity around the fish due to the walls in a confined space) because the maximum cross-section area of the fish was less than 10% of that of the respiromenter (Bell & Terhune 1970).

*Metabolic traits*

Following the acclimation period (i.e. 1 h at 1.0 cm s$^{-1}$), oxygen consumption of individual fish swimming under each velocity in the respirometer was recorded over 20 min. This time period (i.e. 20 min) is enough to detect reduction of oxygen concentration (>5% decrease of saturation) in the water (Plaut 2000). Dissolved oxygen was measured inside the respirometer using a fibre optic oxygen instrument (Witrox 1, Loligo$^{®}$ Systems, Denmark), which includes a Pt1000 temperature sensor and software for compensation of oxygen data to changes in temperature or barometric pressure in real-time. For calibration, we used air-saturated water from the buffer tank as 100% saturation and a solution of sodium sulfate (0.141 M) as 0%. The water in the respirometer was supplied from the buffer tank while slowly increasing the velocity, to increase dissolved oxygen concentration at 100% saturation. Blank-respirometer measurements (i.e. with no fish) were carried out for 10 min after the swimming performance of each fish had been done, for possible corrections for microbial metabolism.

Linear regression analysis of oxygen reduction over time was computed for individual fish under each velocity and the slope or regression coefficient ($c$, % s$^{-1}$) was used to calculate oxygen consumption ($V_{O_2}$, mg O$_2$ h$^{-1}$) as:

$$V_{O_2} = c \times 3600 \times S_{O_2} \times V$$

where the value 3600 converts seconds to hours, $S_{O_2}$ is the oxygen concentration (mg L$^{-1}$) at 100% saturation in the trial, and $V$ is the volume (i.e. 0.17 L) of the closed respirometer (Green & Carritt 1967). Atmospheric pressure on the oxygen probe was adjusted based on the actual pressures before measuring oxygen consumption. Background (microbial) oxygen consumption in the blank was subtracted from the measurements (Keys 1930). The oxygen concentration in the respirometer during the tests was always greater than 7.0 mg L$^{-1}$ to avoid stress effects due to hypoxia on physiological processes (Blaikie & Kerr 1996). Each mosquitofish was used only once in the trial, measured (TL, ±1 mm) and weighed (WW, ±0.1 mg) after finishing the experiment. All measurements of $U_{crit}$ and oxygen consumption were performed during the daytime (around 12:00 solar time).

For individual fish, $V_{O_2}$ was used as an index of metabolic rate (MR). Maximal metabolic rate (MMR) was obtained as the highest MR observed at the different swimming

velocities (generally close to $U_{crit}$). Resting metabolic rate (RMR) was estimated by using the MRs measured at different swimming velocities to establish a regression equation, $MR = ue^{vS}$, describing the relationship between MR and swimming velocity ($S$), and estimating RMR as $u$, i.e. extrapolating to a swimming velocity of 'zero' (Brett 1964; Xie & Sun 1990). Factorial aerobic scope (FAS), which indicates the ability of fish to respond to environmental extremes or other challenges (e.g. swimming performance under variable flow conditions), was calculated as the ratio MMR/RMR (Weibel & Hoppeler 2005; Killen *et al.* 2007).

*Data analyses*

All statistical analyses were performed in the R statistical software (R Core Team 2014). The significance level was set at $\alpha = 0.05$. We $\log_{10}$-transformed all variables (TL, WW, $U_{crit}$, MMR, RMR, FAS) to satisfy the assumptions of the parametric statistical methods (i.e. normality, homoscedasticity and linearity). We used analysis of covariance (ANCOVA) to test for differences in the response variables between sexes, as the categorical factor, after accounting for the effects of the covariate (generally fish weight, WW). We first tested for interactions between the covariate and the categorical factor: if these interactions are significant, they indicate that the slopes are not homogeneous and thus the parallelism assumption of the standard ANCOVA is not satisfied, but they also imply effects of the terms involved, even if the factors alone are not significant (García-Berthou & Moreno-Amich 1993). When the interaction was non-significant, it was removed from the model to improve statistical power and a standard ANCOVA design was used (i.e. homogeneous slopes were assumed). Finally, the model tested differences between sexes with WW as the covariate, which is equivalent to comparing intercept or mean values of the response variables adjusted to the mean weight (García-Berthou & Moreno-Amich 1993).

# Results

## Study 1: World distribution and introduction correlates of mosquitofishes

*Discrepancy among databases*

The four databases reviewed contained many clear errors. For example, they cited *G. affinis* in Spain, Portugal, France, Hungary and Greece, whereas clearly only *G. holbrooki* present in these countries based on genetic studies (Vidal *et al.* 2010). In Australia, mosquitofish were introduced from the USA to mainland Sydney in 1925 (Wilson 1960) and Lloyd & Tomasov (1985) provided clear evidence on gonopodium morphology for *G. holbrooki*, whereas *G. affinis* is cited in DIAS, FishBase and GBIF. There were strong differences in the number of countries where *G. holbrooki* and *G. affinis* have established; there was more disagreement for *G. holbrooki* than for *G. affinis* (Table 5).

Table 5: Number of countries where *G. holbrooki* and *G. affinis* have established (excluding territories and islands that are not whole countries). We use ranges in our estimates because of two uncertain but likely countries for *G. holbrooki*, and 6 for *G. affinis*. DIAS: Database on Introduction of Aquatic Species of Food and Agriculture Organization; GISD: Global Invasive Species Database of the IUCN's Invasive Species Specialist Group (ISSG); and GBIF: Global Biodiversity Information Facility.

| Databases | www hyperlink | No. countries where *G. holbrooki* have established | No. countries where *G. affinis* have established |
|---|---|---|---|
| FishBase | www.fishbase.org | 24 | 57 |
| FAO's DIAS | www.fao.org/fishery/dias | 21 | 50 |
| GISD, ISSG | www.issg.org/database/welcome | 17 | 58 |
| GBIF | http://data.gbif.org | 32 | 53 |
| Estimate of this study | | 49–51 | 44–50 |

The two *Gambusia* species have established in all continents except Antarctica, but *G. holbrooki* is present mainly in southern Europe, the Middle East, northern and western Africa, western Asia and Australia, and *G. affinis* is present mainly in the Americas, southern Africa and eastern Asia (Fig. 10). Since the 1900s, we estimated that the two *Gambusia* species have been collectively introduced to 113 countries outside their natural geographical ranges. *G. holbrooki* has established in 49–51 countries (2 are uncertain but

likely *G. holbrooki*) and *G. affinis* has established in 44–50 countries (6 are uncertain but likely *G. affinis*). According to our review, the two species have established in the following countries:

- **Countries with *G. holbrooki* established:** Afghanistan, Albania, Armenia, Australia, Azerbaijan, Bosnia and Herzegovina, Bulgaria, Chile, Croatia, Cyprus, Egypt, Eritrea, Ethiopia, France, Georgia, Greece, Hungary, India, Islamic Republic of Iran, Italy, Iraq, Kazakhstan, Kenya, Kyrgyzstan, Lebanon, Madagascar, Malaysia, Mauritius, Morocco, Papua New Guinea, Portugal, Romania, Russian Federation, Saudi Arabia, Slovenia, Spain, Sudan, Syrian Arab Republic, Tajikistan, Tunisia, Turkey, Turkmenistan, Ukraine, Uzbekistan, Republic of Macedonia, United Arab Emirates, Viet Nam, and Yemen. Uncertain species but likely *G. holbrooki* for Libya and Somalia.

- **Countries with *G. affinis* established:** Argentina, Bangladesh, Bolivia, Botswana, Cambodia, Canada, Cape Verde, Chile, China, Democratic Republic of Congo, Dominican Republic, East Timor, Ecuador, Federated States of Micronesia, Fiji, Haiti, India, Israel, Italy, Japan, Jordan, Laos PDR, Marshall Islands, Mozambique, Myanmar, Nauru, Nepal, New Zealand, Pakistan, Palau, Peru, Philippines, Samoa, Singapore, Solomon Islands, South Africa, Sri Lanka, State of Palestine, Syrian Arab Republic, Taiwan, Thailand, Vanuatu, Viet Nam, Zambia, and Zimbabwe. Uncertain species but likely *G. affinis* for Central African Republic, Democratic Congo, Kiribati, Tanzania, The Bahamas.

They also have established in territories and islands that are not whole countries as following:

- **Islands with *G. holbrooki* established:** Bermuda (UK), Canary Islands (Spain), Christmas Island (Australia), Corsica (France), Krk Island (Croatia), New Ireland (Papua New Guinea), Puerto Rico (USA), Réunion (France), Rodrigues (France), Tahiti (French Polynesia), and Tasmania (Australia).

- **Islands with *G. affinis* established:** American Samoa (USA), Caroline Islands (Micronesia), Cook Islands (New Zealand), French Polynesia (France), Guam (USA), Hainan (China), Hong Kong (China), Krusadai Island (India), New Caledonia (France), Northern Marianas Islands (USA), Pagan Islands (Northern Marinas Islands), and Puerto Rico (USA).
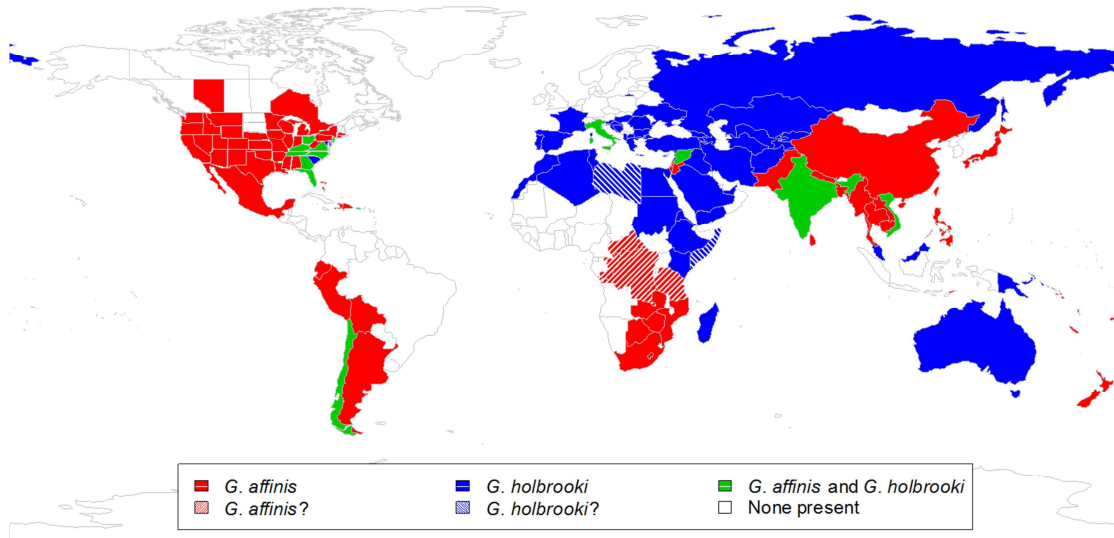
Figure 10: World map of the current distributions of *G. holbrooki* and *G. affinis*. "*G. holbrooki*?" and "*G. affinis*?" indicate that the species is uncertain but is likely *G. holbrooki* and *G. affinis*, respectively.

*History of introductions*

Although there were no significant differences in the number of donations per country between *G. holbrooki* and *G. affinis* (unequal variance *t*-test, $P = 0.464$; Levene's test, $P = 0.197$; $n = 392$), date of first introduction to the country was significantly different (*t*-test, $P = 0.005$; Levene's test, $P = 0.0019$; $n = 108$). AIC values and significance tests showed that the relationship between number of donations and date of first introduction of the two species yielded different GAM models and the smoothers were clearly significant for *G. holbrooki* ($P < 0.0005$, $R^2_{adj.} = 0.173$, $n = 58$), but not for *G. affinis* ($P = 0.146$, $R^2_{adj.} = 0.195$, $n = 50$). The donation numbers varied with year, with donations of *G. holbrooki* peaking in the 1920s and donations of *G. affinis* showing a flatter response (Fig. 11). Overall, although the total number of introductions of the two species is similar, *G. holbrooki* was introduced earlier (on average) with a peak of intensity in the 1920s, whereas the introductions of *G. affinis* are dominant in the second half of the XXth century and constant through time. However, there was no relationship between establishment probability and date of first introduction to the country either for *G. holbrooki* ($P = 0.999$, $n = 58$) and *G. affinis* ($P = 0.128$, $n = 50$).
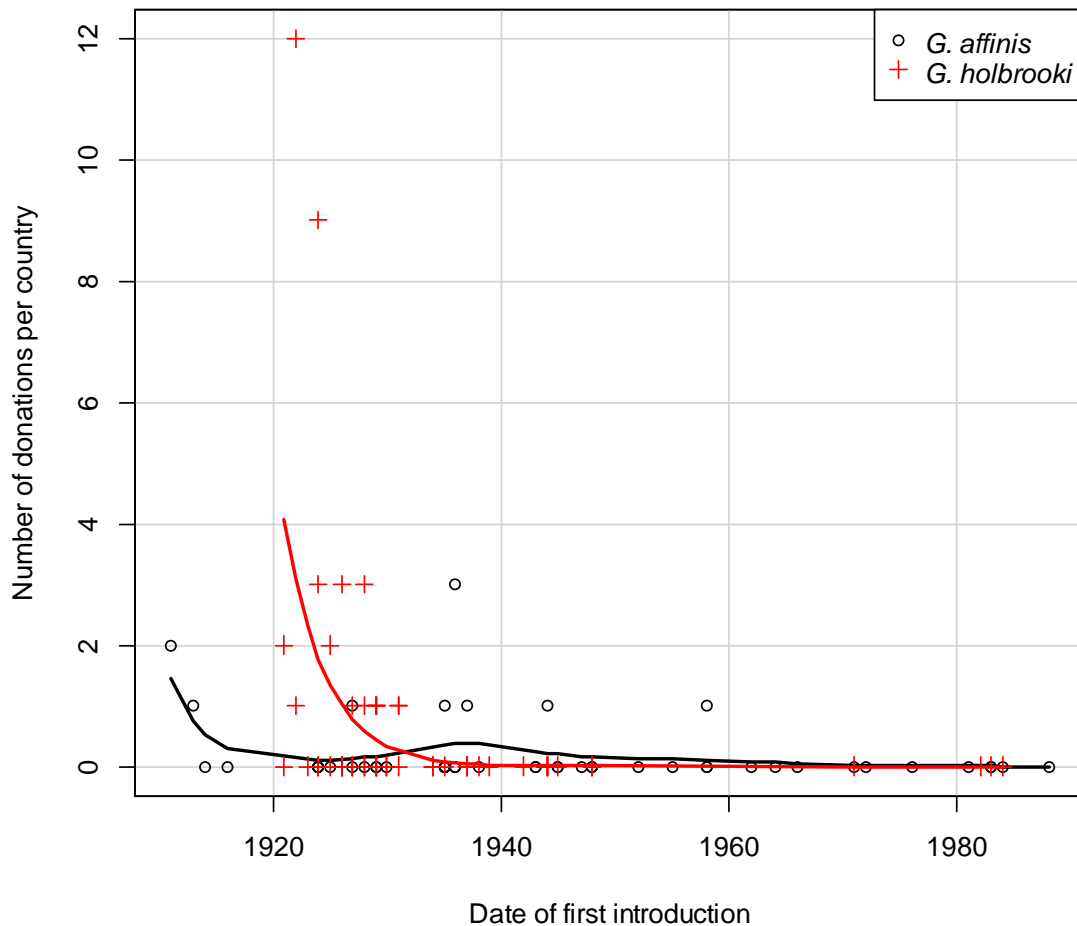
Figure 11: Generalized additive models for the relationship between number of donations per country (number of times that the species has been introduced from the different countries) and date of first introduction to the country of *G. holbrooki* and *G. affinis*. Red curve denotes *G. holbrooki* ($P < 0.0005$, $R^2_{adj.} = 0.173$, deviance explained $= 39.3\%$, $n = 58$ countries) and black curve denotes *G. affinis* ($P = 0.146$, $R^2_{adj.} = 0.195$, deviance explained $= 30.5\%$, $n = 50$ countries.

There were clear introduction pathways for the two species, where *G. holbrooki* was introduced to Spain in 1921 then quickly into southern Europe and then continued to spread to some countries in the Middle East, and northern and eastern Africa (Fig. 12). By contrast, *G. affinis* first went to Hawaii, then was widely spread to Pacific islands and then to Asia. *G. affinis* also went to South America (i.e. Chile and Argentina) and southern Africa (i.e. Zimbabwe) then spread to neighbouring countries (Fig. 13).
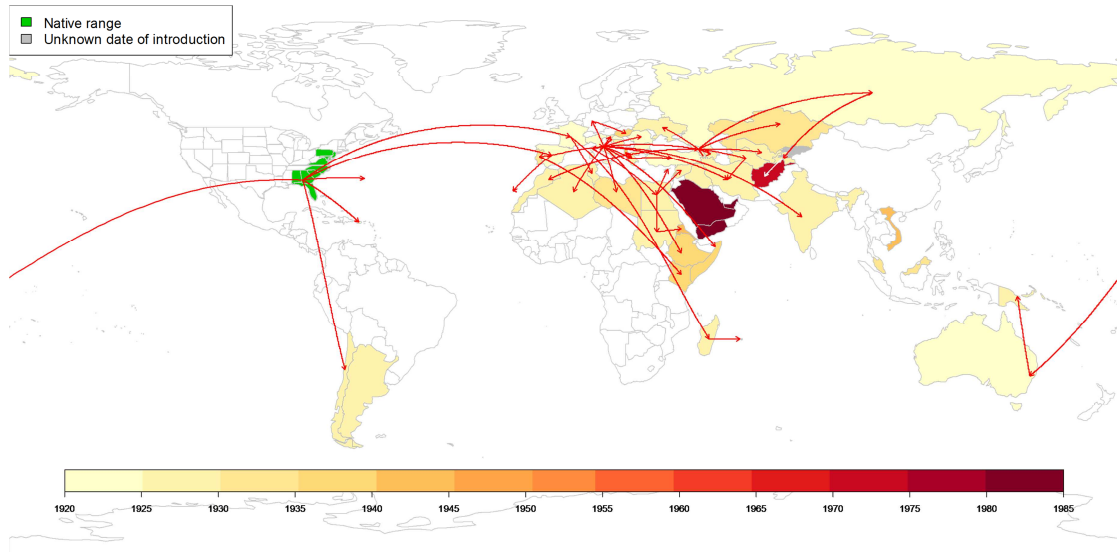
Figure 12: World map of the history of introductions of *G. holbrooki*: routes and dates of first introduction. Arrow lines on the map indicate known routes of introduction from an originating country to a receiving country. Colours on the map indicate date of first introductions except in the USA; white colour countries denote that *G. holbrooki* is not established in the country.
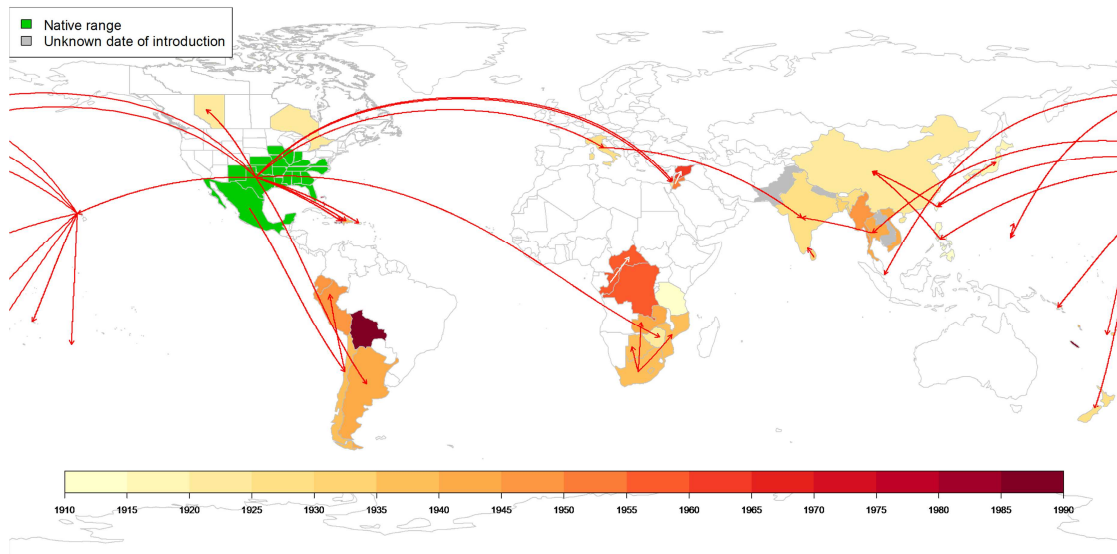


Figure 13: World map of the history of introductions of *G. affinis*: routes and dates of introduction. Arrow lines on the map indicate known routes of introduction from an originating country to a receiving country. Colours on the map indicate date of first introductions except in the USA and Mexico; white colour countries denote that *G. affinis* is not established in the country.

The mean distance of introductions was significantly longer for *G. affinis* than for *G. holbrooki* (*t*-test, *n* = 73, *P* < 0.05). The mean dispersion direction was significantly different between the two species (*U* = 0.482, *n* = 61, *P* < 0.001), with the distribution for *G. holbrooki* being clearly unimodal (*Z* = 0.598, *n* = 61, *P* < 0.001; Fig. 14) with an average of 37.8 ± 5.8° (northeast direction), and the distribution for *G. affinis* being more uniform (*Z* = 0.138, *n* = 57, *P* = 0.071; Fig. 15). The directions did not depend significantly on the year of introduction for both *G. holbrooki* (*P* = 0.306; Fig. 16) and *G. affinis* (*P* = 0.260; Fig. 17).
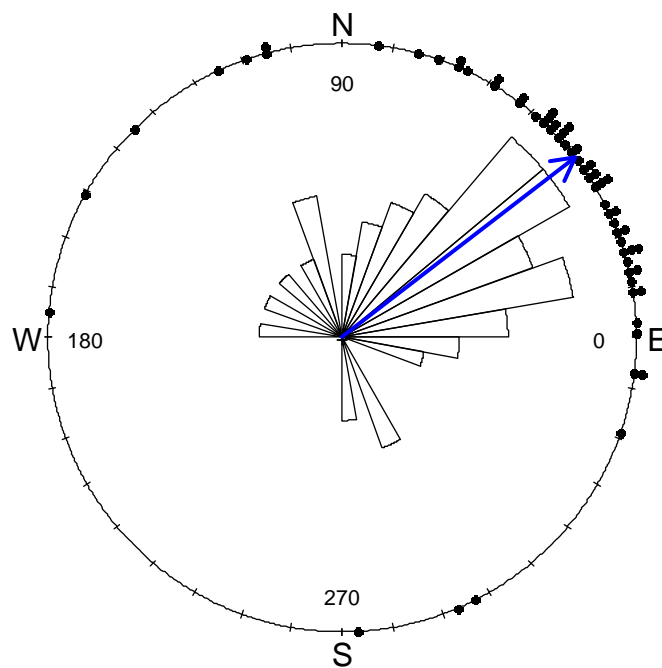


Figure 14: Rose diagram showing the direction of introductions for *G. holbrooki*. The blue arrow indicates the mean direction.
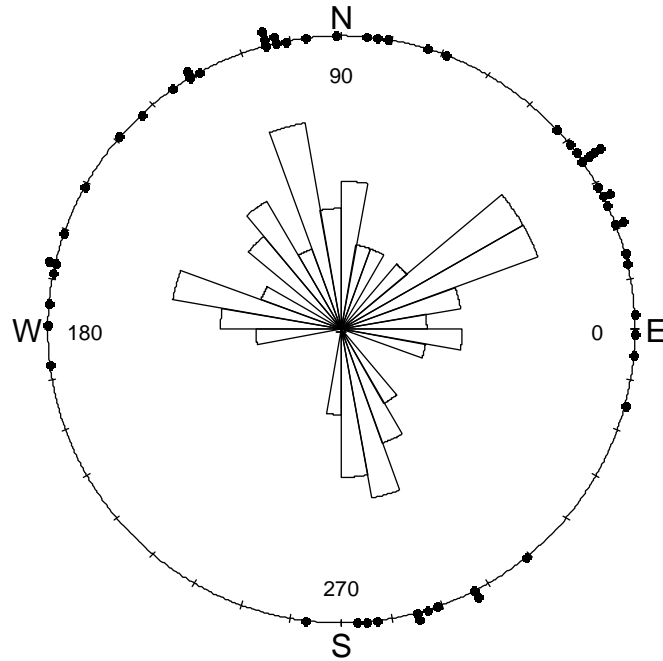
Figure 15: Rose diagram showing the direction of introductions for *G. affinis.*
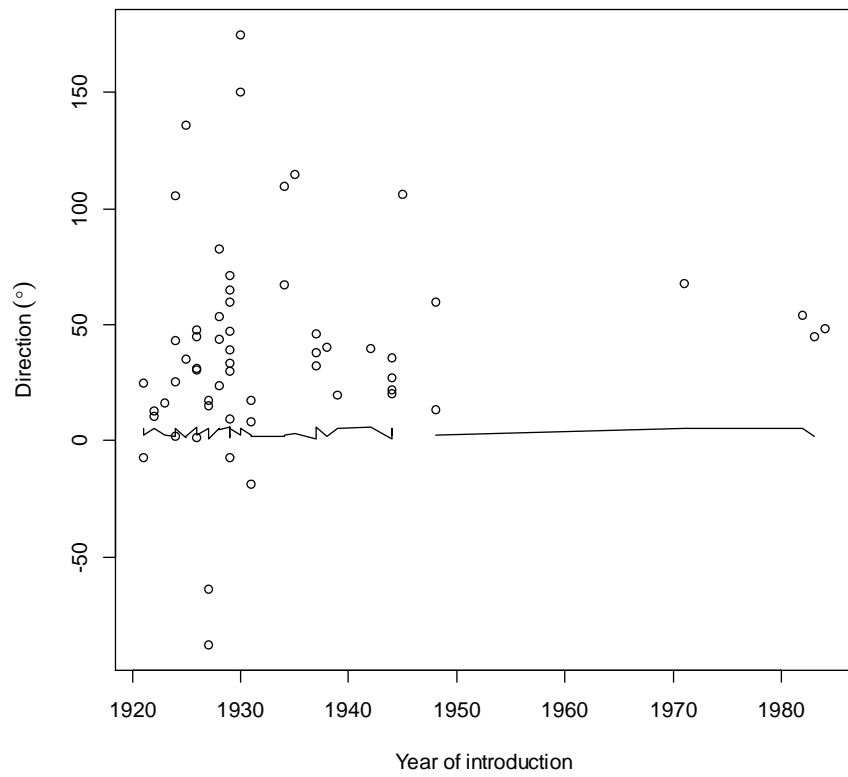


Figure 16: Circular-linear regression model for the relationship between introduction direction and year of first introduction to the country for *G. holbrooki* ($P > 0.15$, $n = 58$). Negative directions are southward.
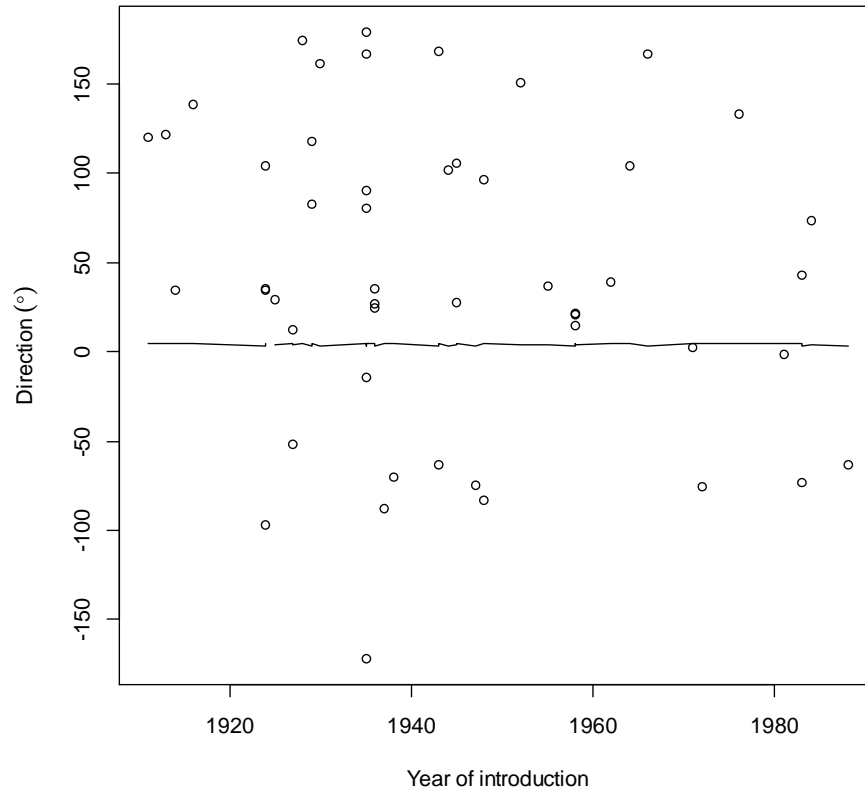
Figure 17: Circular-linear regression model for the relationship between introduction direction and year of first introduction to the country for *G. affinis* (*P* > 0.24, *n* = 50). Negative directions are southward.

*Introduction correlates*

Variable importance plots for predictor variables from random forests (Fig. 18) showed that the RECEIVING COUNTRY and ESTABLISHMENT of *G. holbrooki* and *G. affinis* were mostly related to climate, latitude and longitude, whereas ORIGINATING COUNTRY and INTRODUCTION DATE were mostly related to country areas, economics and also climate. However, the relative importance of the 12 predictor variables varied for the two *Gambusia* species.

The RECEIVING COUNTRY was related to frost-day frequency, longitude and precipitation for *G. holbrooki* (Fig. 18a), and latitude, longitude and population density for *G. affinis* (Fig. 18b). Longitude was important predictor for both species. Therefore, *G. holbrooki* was mostly introduced to countries with low frost-day frequency and precipitation and *G. affinis* was mostly introduced to lower latitudes, whereas both species spread from low to higher longitude.

The ᴇꜱᴛᴀʙʟɪꜱʜᴍᴇɴᴛ was mostly related to latitude, frost-day frequency and precipitation for *G. holbrooki* (Fig. 18c), and mean, minimum temperatures and frost-day frequency for *G. affinis* (Fig. 18d), suggesting that both species have mostly established under warm temperature conditions and less frost-day frequency.

The ᴏʀɪɢɪɴᴀᴛɪɴɢ ᴄᴏᴜɴᴛʀʏ was mostly related to country areas, longitude and GDPC for *G. holbrooki* (Fig. 18e), and GDPC, exports and precipitation for *G. affinis* (Fig. 18f). For *G. holbrooki*, smaller countries were more likely to act as a ᴏʀɪɢɪɴᴀᴛɪɴɢ ᴄᴏᴜɴᴛʀʏ (e.g. out of 50 introductions, 12 were from Italy and 9 from Georgia), and few donations came from larger countries (e.g. 6 from the USA), whereas for *G. affinis*, over half (19 out of 35) came from the USA, which has a high GDPC with low percentage of exports (relative to gross domestic product).

The ɪɴᴛʀᴏᴅᴜᴄᴛɪᴏɴ ᴅᴀᴛᴇ was mostly related to minimum and mean temperatures for both species, whereas country area was the most important predictor for *G. affinis* (Fig. 18g, 18h). Both species were recently introduced to warmer temperature conditions, while *G. affinis* was introduced from large to smaller countries.
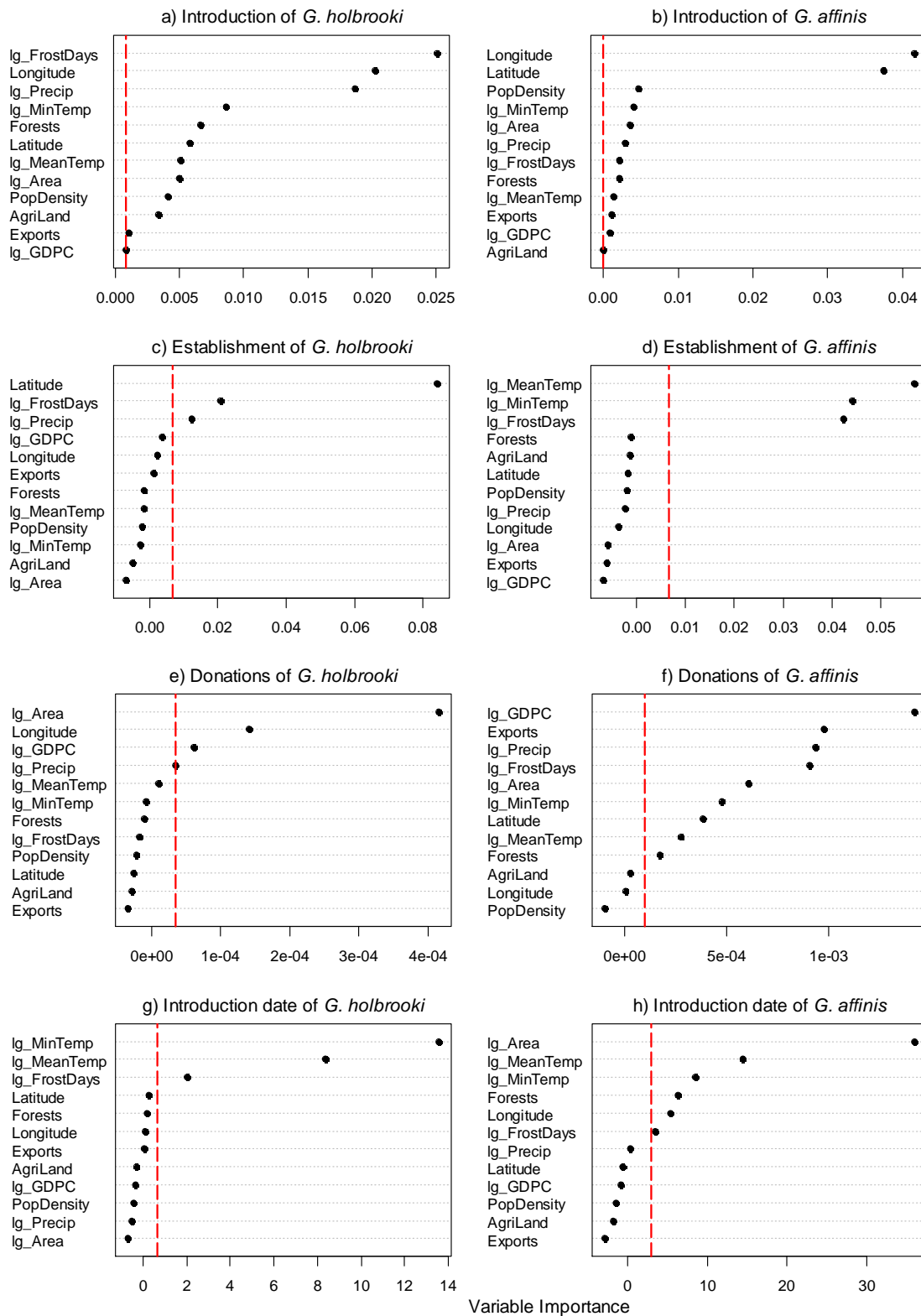
Figure 18: Variable importance plots for predictor variables from random forests: introduction probability of a) *G. holbrooki* and b) *G. affinis*; establishment probability of c) *G. holbrooki* and d) *G. affinis*; donation numbers (number of introductions from a given

country) of e) *G. holbrooki* and f) *G. affinis*; and first-introduction date of g) *G. holbrooki* and h) *G. affinis*. Red dashed lines are the absolute value of the lowest ranking predictor (predictors to right of dashed vertical lines are significant) and higher values of mean decrease in accuracy on x-axis indicate greater importance to the classification. Predictors are as follows: AgriLand is the percentage of areas of agricultural land; lg_Area is the total area of the country (km$^2$; $\log_{10}$ transformed); Exports is the percentage of export as gross domestic product; Forests is the percentage of areas of forest coverage; lg_Frost.Days is the frost day frequency (days; $\log_{10}(x + 1)$); lg_GDPC is the gross domestic product per capita (US$; $\log_{10}$ transformed); lg_MeanTemp is the daily mean temperature (degree C; $\log_{10}(x + \text{minimum})$ transformed); lg_MinTemp is the daily minimum temperature (degree C; $\log_{10}(x + \text{minimum})$ transformed); PopDensity is the number of inhabitants per km$^2$; lg_Precip is the daily precipitation (mm/day; $\log_{10}$ transformed); Latitude and Longitude are in degrees.

**Study 2: Meta-analysis of the ecological impact of mosquitofishes**

*Differences among biota groups*

The mean effect sizes for three of the nine biota groups (fish, amphibians and macroinvertebrates) were significantly smaller than zero (Fig. 19), indicating general effects of mosquitofish presence. The mean effect sizes differed significantly among the nine groups ($Q_M = 38.650$, d.f. $= 8$, $P < 0.0001$). Although the mean effect sizes for zooplankton, phytoplankton, and macrophytes were not significantly different from zero, heterogeneity for each group was significant ($P < 0.0001$) and large (Table 7). There was neither a significant effect nor heterogeneity ($P > 0.05$) for picoplankton, periphyton and bacteria (Fig. 19). There was high heterogeneity among studies for fish and amphibians (Table 6), because total variation across studies differed significantly among variable types ($Q_M = 18.584$, d.f. $= 5$, $P = 0.002$ for fish; $Q_M = 24.955$, d.f. $= 6$, $P = 0.0003$ for amphibians) and between amphibian taxonomic groups ($Q_M = 13.791$, d.f. $= 1$, $P = 0.0002$).
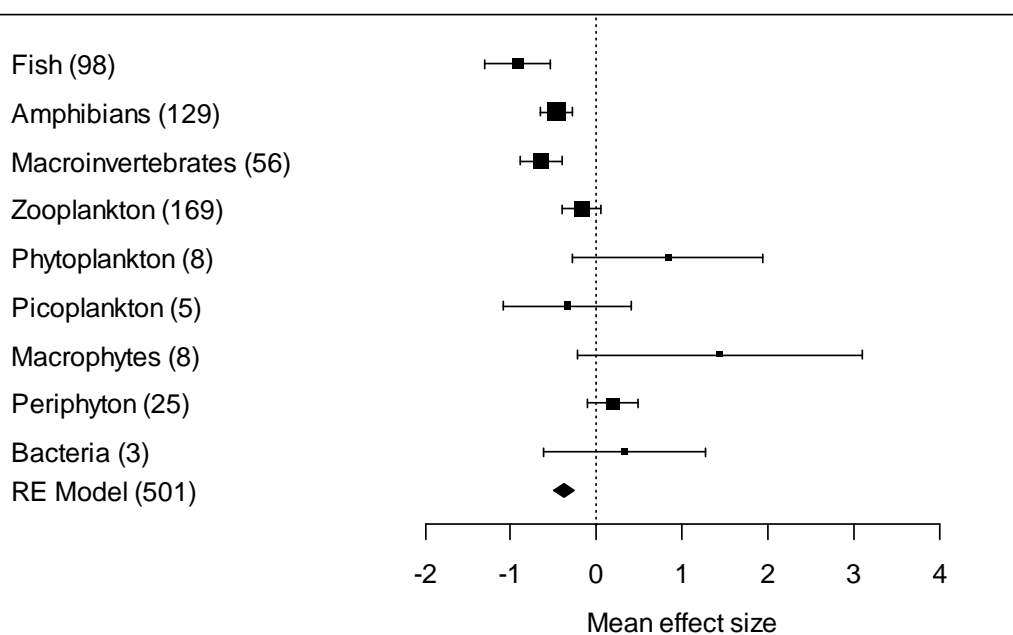


Figure 19: Forest plot of mean effect sizes (Hedges' *d*) of the effects of mosquitofish on biota groups (detailed values in Table 7). The bars around the mean denote 95% confidence intervals based on a random-effects (RE) model: a mean effect size is significantly different from zero when its 95% confidence interval does not include zero.

Sample sizes for each group are given in parentheses and the area of each square is proportional to the weight of the group in the meta-analysis. Negative mean effect sizes indicate negative effects of mosquitofish on the measured response variable between treatment and control groups (no mosquitofish or only native fish species), and vice versa.

By taxonomic group, mosquitofish negatively affected: Cyprinidae, Poeciliidae, Galaxiidae and Cyprinodontidae among fish; Anura and Urodela for amphibians (Fig. 20; see Table 6 for $I^2$ of each group); Crustacea, Diptera and other invertebrates (benthos and unspecified invertebrates) for macroinvertebrates (Fig. 21); and Copepoda (both Cyclopoida and Calanoida) and other groups (including unspecified microcrustaceans, and crustacean nauplii) for zooplankton (Fig. 22). Overall effect size for Cladocera (density/biomass, size/stage, and life history) was not significantly different from zero but density/biomass alone was (mean $d = -0.82$; CI = -1.21, -043; $k = 32$). Mosquitofish had significantly positive effects on Rotifera (Fig. 22).
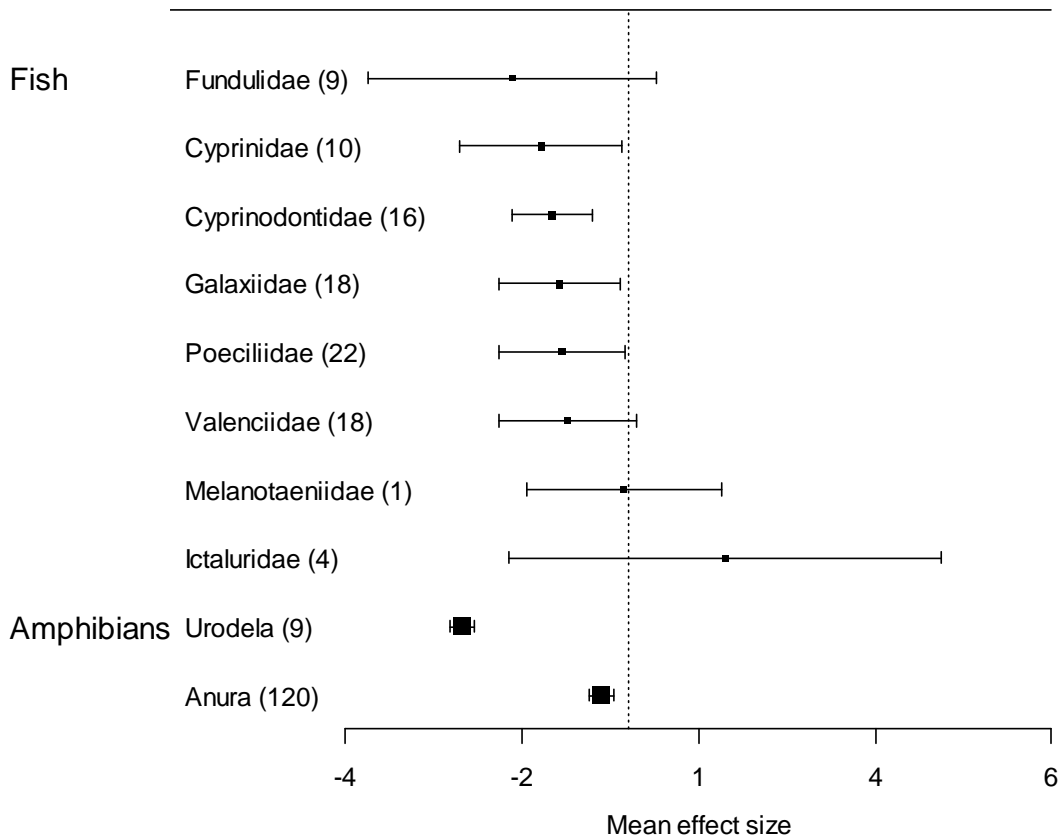


Figure 20: Forest plot of mean effect sizes (Hedges' $d$) of the effects of mosquitofish on taxonomic groups of fish and amphibians (detailed values in Table 6). The bars around the mean denote 95% confidence intervals based on a random-effects model: a mean effect

size is significantly different from zero when its 95% confidence interval do not include zero. Sample sizes for each group are given in parentheses and the area of each square is proportional to the weight of the group in the meta-analysis. Negative mean effect sizes indicate negative effects of mosquitofish on the measured response variable between treatment and control groups (no mosquitofish or only native fish species), and vice versa.

Table 6: Mean effect sizes (*d*), 95% confidence intervals (CI; significant results are in bold), sample size (*k* = number of case studies), and residual heterogeneity ($I^2$: percentage of total variance across studies due to heterogeneity) for each taxonomic group of fish and amphibians.

| Group | Taxonomic group | *d* | 95% CI | *k* | $I^2$ (%) |
|---|---|---|---|---|---|
| Fish | Fundulidae | -1.64 | -3.68, 0.40 | 9 | 87.59 |
| | Cyprinidae | **-1.23** | **-2.37, 0.08** | 10 | 88.08 |
| | Cyprinodontidae | **-1.08** | **-1.64, -0.51** | 16 | 71.98 |
| | Galaxiidae | **-0.97** | **-1.83, -0.11** | 18 | 82.37 |
| | Poeciliidae | **-0.93** | **-1.83, -0.03** | 22 | 86.63 |
| | Valenciidae | -0.86 | -1.83, 0.11 | 18 | 89.11 |
| | Melanotaeniidae | 0.05 | -1.44, 1.33 | 1 | 0.00 |
| | Ictaluridae | 1.37 | -1.69, 4.43 | 4 | 93.37 |
| | Fish overall | **-0.93** | **-1.30, -0.55** | 98 | 85.69 |
| Amphibians | Urodela | **-2.35** | **-3.76, -0.95** | 9 | 82.32 |
| | Anura | **-0.38** | **-0.54, -0.21** | 120 | 46.90 |
| | Amphibians overall | **-0.47** | **-0.65, -0.29** | 129 | 54.18 |
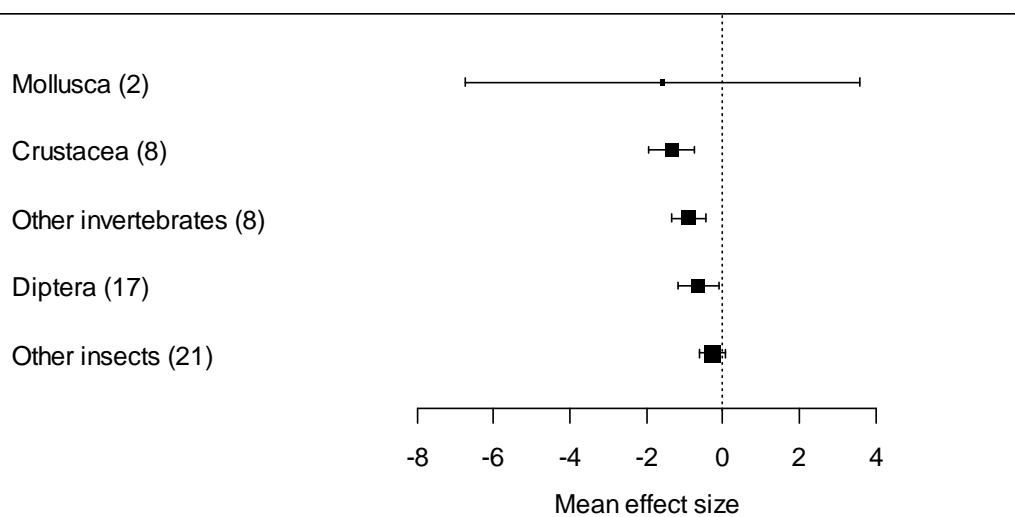
Figure 21: Forest plot of mean effect size (Hedges' *d*) of the effects of mosquitofish on taxonomic groups of macroinvertebrates. The bars around the mean denote 95% confidence intervals based on a random-effects model. A mean effect size is significantly different from zero when its 95% confidence interval do not include zero. Sample sizes for each group are given in parentheses and the area of each square is proportional to the weight of the group in the meta-analysis. Negative mean effect sizes indicate negative effects of mosquitofish on the measured response variable between treatment and control groups (no mosquitofish or only native fish species), and vice versa.
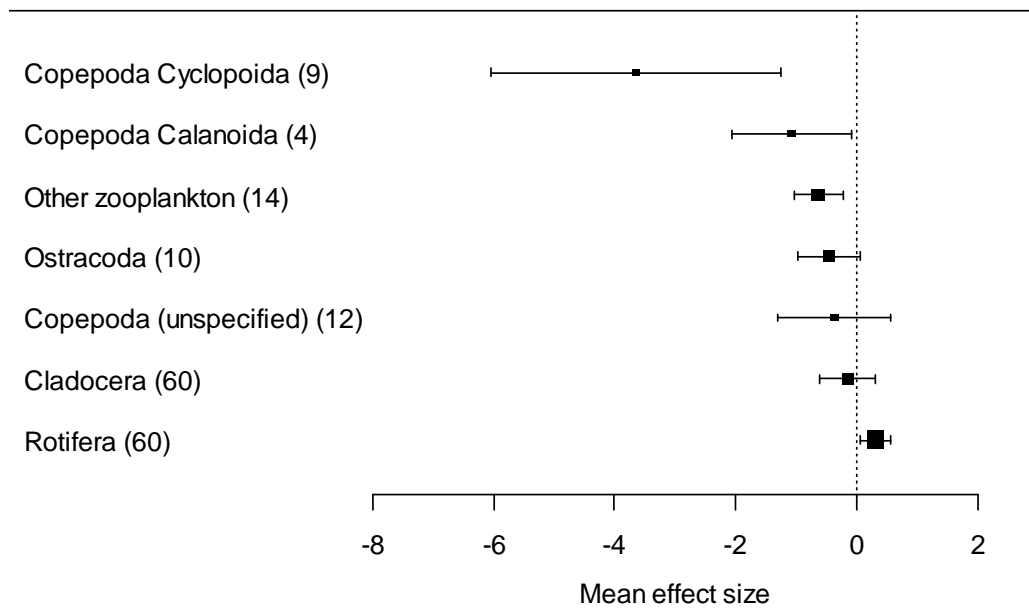


Figure 22: Forest plot of mean effect size (Hedges' *d*) of the effects of mosquitofish on taxonomic groups of zooplankton. The bars around the mean denote 95% confidence intervals based on a random-effects model: a mean effect size is significantly different from zero when its 95% confidence interval do not include zero. Sample sizes for each group are given in parentheses and the area of each square is proportional to the weight of the group in the meta-analysis. Negative mean effect sizes indicate negative effects of mosquitofish on the measured response variable between treatment and control groups (no mosquitofish or only native fish species), and vice versa.

*Differences among variable types*

The mean effect sizes of the variable types differed significantly among groups (Table 7; $Q_M = 33.88$, d.f. = 9, $P < 0.0001$). Mosquitofishes generally increased the number of

agonistic behaviour received by native fish and amphibians, and reduced the density/biomass of native fish, amphibians and zooplankton (Table 7, Fig. 23). By contrast, there were no significant overall effects for size/stage structure, life history, diversity, feeding and other behaviour (Fig. 23), concentration of chemicals, and transparency other than the other physical and chemical properties (i.e. conductivity, DO, pH; Table 7), although heterogeneity across studies for each group was high (Table 7). Diversity metrics significantly decreased for macroinvertebrates and increased for phytoplankton in presence of mosquitofish (Table 7).
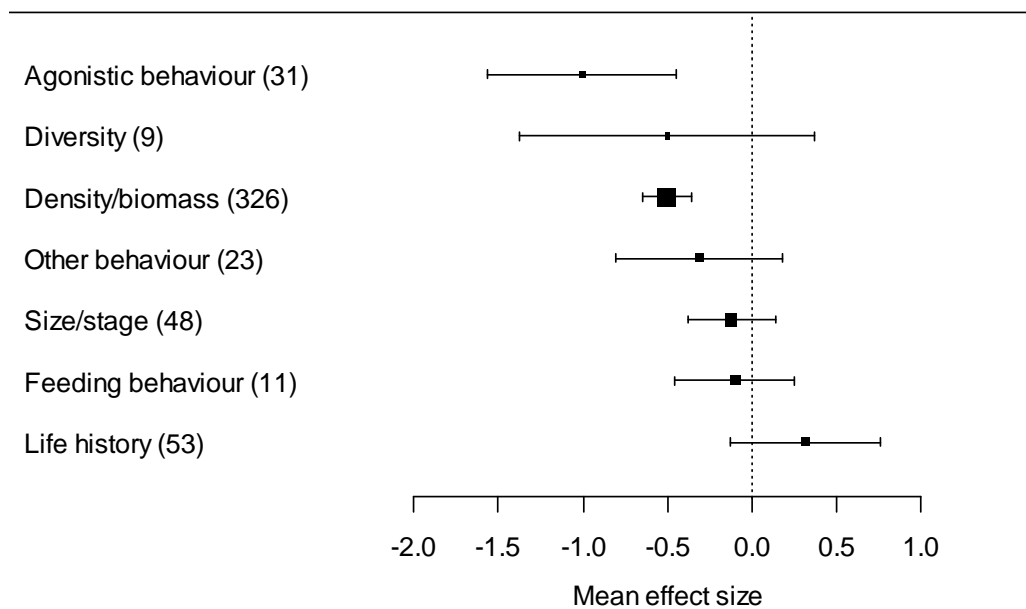


Figure 23: Forest plot mean effect size (Hedges' *d*) of the effects of mosquitofish on variable types of biota. The bars around the mean denote 95% confidence intervals based on a random-effects model: a mean effect size is significantly different from zero when its 95% confidence interval do not include zero. Sample sizes for each group are given in parentheses and the area of each square is proportional to the weight of the group in the meta-analysis. Negative mean effect sizes indicate negative effects of mosquitofish on the measured response variable between treatment and control groups (no mosquitofish or only native fish species), and vice versa.

Table 7: Mean effect sizes (*d*), 95% confidence intervals (CI; significant results are in bold), sample size (*k* = number of case studies), and residual heterogeneity ($I^2$: percentage of total variance across studies due to heterogeneity) for each variable type of biota and ecosystem feature.

| Group | Variable type | *d* | CI | *k* | $I^2$ (%) |
|---|---|---|---|---|---|
| Fish | Density/biomass | **-2.17** | **-3.14, -1.19** | 39 | 92.78 |
| | Size/stage | -0.16 | -0.96, 0.63 | 12 | 76.83 |
| | Life history | 0.01 | -1.07, 1.08 | 6 | 72.17 |
| | Feeding behaviour | -0.25 | -0.70, 0.20 | 7 | 0.00 |
| | Agonistic behaviour | **-0.96** | **-1.58, -0. 34** | 28 | 82.12 |
| | Other behaviour | 0.67 | -0.01, 1.35 | 6 | 60.51 |
| | Fish overall | **-0.93** | **-1.30, -0.55** | 98 | 85.69 |
| Amphibians | Density/biomass | **-0.97** | **-1.32, -0.62** | 54 | 65.51 |
| | Size/stage | -0.11 | -0.39, 0.18 | 27 | 29.54 |
| | Life history | 0.06 | -0.29, 0.40 | 23 | 32.01 |
| | Diversity | -0.31 | -1.29, 0.68 | 1 | 0.00 |
| | Feeding behaviour | 0.14 | -0.43, 0.71 | 4 | 0.00 |
| | Agonistic behaviour | **-1.68** | **-2.54, -0.81** | 3 | 0.00 |
| | Other behaviour | **-0.69** | **-1.21, -0.17** | 17 | 67.66 |
| | Amphibians overall | **-0.47** | **-0.65, -0.29** | 129 | 53.86 |
| Macroinvertebrates | Density/biomass | **-0.60** | **-0.86, -0.34** | 52 | 37.35 |
| | Size/stage | -0.50 | -1.40, 0.39 | 2 | 0.00 |
| | Diversity | **-1.82** | **-3.48, -0.17** | 2 | 65.01 |
| | Macroinvertebrates overall | **-0.64** | **-0.89, -0.40** | 56 | 38.37 |
| Macrophytes | Density/biomass | 1.43 | -0.26, 3.08 | 8 | 84.08 |
| Zooplankton | Density/biomass | **-0.29** | **-0.49, -0.09** | 133 | 55.95 |
| | Size/stage | -0.45 | -1.69, 0.80 | 7 | 86.59 |
| | Life history | 0.67 | -0.18, 1.51 | 24 | 93.94 |
| | Diversity | **-0.39** | **-1.40, 0.61** | 5 | 59.91 |
| | Zooplankton overall | -0.17 | -0.39, 0.05 | 169 | 75.46 |
| Phytoplankton | Density/biomass | 0.70 | -0.53, 1.93 | 7 | 77.46 |
| | Diversity | **1.77** | **0.13, 3.41** | 1 | 0.00 |
| | Phytoplankton overall | 0.83 | -0.28, 1.93 | 8 | 75.19 |
| Picoplankton | Density/biomass | -0.34 | -1.09, 0.40 | 5 | 0.00 |
| Periphyton | Density/biomass | 0.19 | -0.11, 0.48 | 25 | 0.00 |
| Bacteria | Density/biomass | 0.33 | -0.62, 1.27 | 3 | 0.00 |
| | Subgroup for biota | **-0.39** | **-0.51, -0.27** | 501 | 72.94 |
| Ecosystem features | Concentration of chemicals | -0.06 | -0.41, 0.29 | 38 | 45.81 |
| | Transparency | -0.67 | -1.50, 0.16 | 18 | 74.99 |
| | Other physical and chemical properties | **0.37** | **0.04, 0.70** | 20 | 0.00 |
| | Subgroup for ecosystem features | -0.06 | -0.30, 0.19 | 76 | 45.28 |
| All | | **-0.34** | **-0.45, -0.23** | 577 | 69.78 |

*Meta-regression*

Meta-regression analyses helped to reduce the amount of unexplained heterogeneity (Table 8). Mean effect sizes for agonistic behaviour of native fish were positively related to depth of the experimental setting (DEP) but negatively related to density of mosquitofish used (DMO), water temperature (TEM) and experimental duration (DUR). The mean effect sizes for density/biomass were related: positively to DEP and negatively to DMO for fish; positively with density of target species used (DTA) and DEP and negatively to DMO and TEM for amphibians; negatively related to DEP and nonlinearly to absolute latitude (LAT) and for macroinvertebrates; and positively related to DEP and also nonlinearly to LAT for zooplankton.

Table 8: Meta-regression models of mean effect sizes for agonistic behaviour of fish ($d_{FA}$) and density/biomass of fish ($d_{FD}$), amphibians ($d_A$), macroinvertebrates ($d_M$) and zooplankton ($d_Z$). Sample size ($k$ = number of case studies), heterogeneity explained by the model ($Q_M$) and its significance (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$), and residual heterogeneity ($I^2$: percentage of total variance across studies due to heterogeneity) is also shown.

| Model | $k$ | $Q_M$ | $P$ | $I^2$ (%) |
|---|---|---|---|---|
| $d_{FA} = 1.19 + 2.63$ DEP $– 6.73$ DMO $– 0.10$ TEM $– 0.06$ DUR | 24 | 12.18 | * | 80.58 |
| $d_{FD} = –5.39 + 11.49$ DEP $– 0.01$ DMO | 26 | 8.42 | * | 81.59 |
| $d_A = 2.06 – 6.47$ DMO $+ 0.80$ DTA $+ 0.55$ DEP $– 0.14$ TEM | 41 | 42.62 | *** | 0.00 |
| $d_M = –45.43 – 2.71$ DEP $– 2.72$ LAT $+ 0.04$ LAT$^2$ | 52 | 17.78 | ** | 17.60 |
| $d_Z = –31.45 + 0.66$ DEP $+ 1.66$ LAT $– 0.02$ LAT$^2$ | 131 | 13.57 | ** | 52.17 |

Note: DMO: density of mosquitofish used (number per litre); DTA: density of target species used (number per litre); DEP: depth of experimental system (in meters); DUR: experimental duration (day); LAT: absolute latitude (degree); TEM: water temperature (°C).

*Publication bias*

A regression test for funnel plot asymmetry showed a significant result ($P < 0.001$) only for the overall dataset (Fig. 24) and for biota alone, and non-significant results for ecosystem features ($P = 0.804$, Fig. 25), suggesting that studies with non-significant results were likely not published for biota. The funnel plots observed were similar in pattern to the previous ecological meta-analysis performed by Maggi *et al.* (2015), suggesting that there is large variation across studies for ecological experiments. The fail-safe number was

27548 which is larger than $5k + 10 = 2895$, where $k$ is number of case studies in our dataset; therefore, the observed results can be a reliable estimate of the true effect (Rosenberg 2005). However, the trim and fill method indicated that there was no missing study on the right side of the funnel plot for overall dataset.
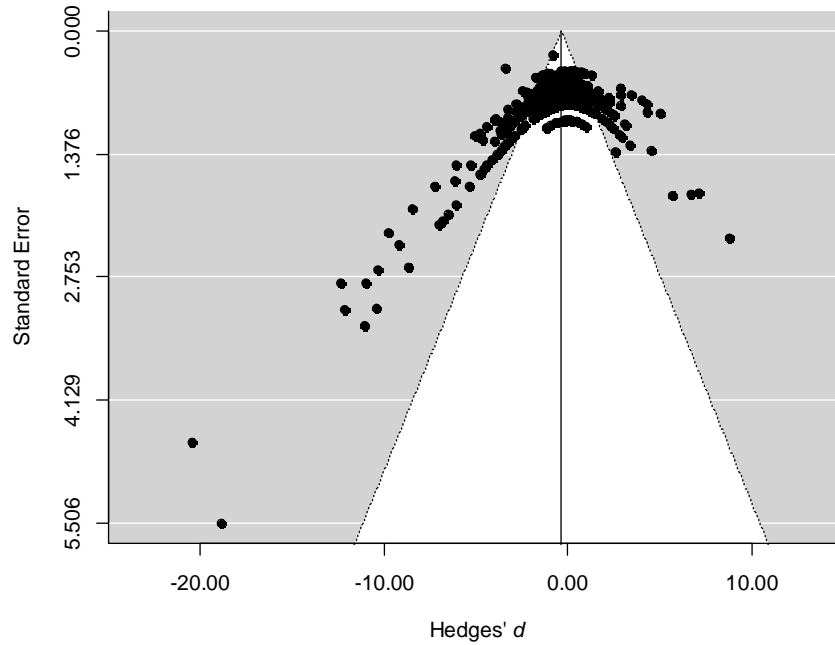


Figure 24: Funnel plot of the effect sizes (Hedges' $d$) with their standard errors for the overall dataset. Egger's asymmetry test: $Z = -11.59$, $P < 0.0001$.
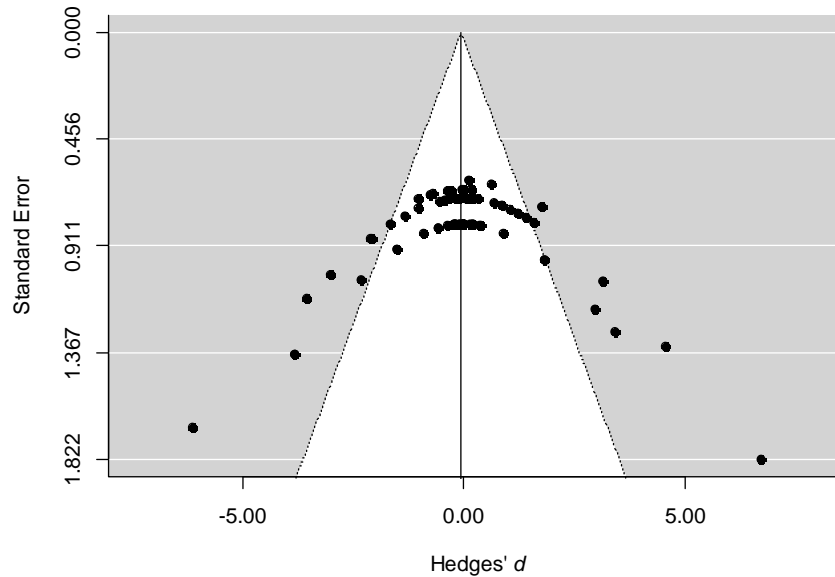


Figure 25: Funnel plot of the effect sizes (Hedges' $d$) with their standard errors for the ecosystem features. Egger's asymmetry test: $Z = -0.25$, $P = 0.804$.

**Study 3: Experiment on the swimming capacity and metabolism of eastern mosquitofish**

According to $r^2$ values, WW was better than TL to predict two response variables per sex ($U_{crit}$ and MMR, see Table 9) and therefore, the former was used for statistical analyses. Three response variables ($U_{crit}$, MMR and RMR) were highly related to overall fish WW, i.e. not accounting for sex (Table 10). All interactions of WW with the categorical factor (i.e. sex) in the ANCOVAs were not significant ($P > 0.05$), indicating no evidence of differences in slopes among groups and that the parallelism assumptions can be assumed for the four response variables (Table 10).

Table 9: Significant linear regression functions ($y = a + bx$) of the critical swimming speed ($U_{crit}$) and maximal metabolic rate (MMR) with TL and WW of mosquitofish by sex. Variables were $\log_{10}$-transformed. *$P < 0.05$; ***$P < 0.001$.

| Sex | Response variable | Independent variable | $a$ | $b$ | $r^2$ | $n$ | $P$ |
|-----|-------------------|----------------------|-----|-----|-------|-----|-----|
| Males | $U_{crit}$ | WW | 1.420 | 0.294 | 0.149 | 30 | * |
| Females | $U_{crit}$ | WW | 1.285 | 0.293 | 0.435 | 30 | *** |
| Males | $U_{crit}$ | TL | –0.191 | 0.962 | 0.137 | 30 | * |
| Females | $U_{crit}$ | TL | 0.032 | 0.726 | 0.355 | 30 | *** |
| Males | MMR | WW | 0.065 | 0.910 | 0.544 | 30 | *** |
| Females | MMR | WW | –0.287 | 0.617 | 0.696 | 30 | *** |

The relationship of $U_{crit}$ with WW differed between sexes (i.e. different intercept for the linear function, see Table 9 and 10), with males having higher $U_{crit}$ values for the same weight range (Fig. 26). Similarly, MMR was significantly higher for males (Fig. 27), after accounting for the effect of weight (Table 10). By contrast, RMR and FAS were not significantly different between sexes (Fig. 28, Table 10).

Table 10: Analyses of covariance (ANCOVAs) of the effect of sex (as the categorical factor) on critical swimming speed ($U_{crit}$), maximal metabolic rate (MMR), resting metabolic rate (RMR) and factorial aerobic scope (FAS), with wet weight (WW) of mosquitofish as the covariate. Variables were $\log_{10}$-transformed. All interactions between the covariate and the categorical factor were non-significant ($P > 0.05$) and they were removed from the model. Consequently, a standard ANCOVA design is reported. **$P <$ 0.01; ***$P < 0.001$.

| Source of variation | $U_{crit}$ | | | MMR | | | RMR | | | FAS | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | $R^2_{adj} = 0.373$ | | | $R^2_{adj} = 0.621$ | | | $R^2_{adj} = 0.263$ | | | $R^2_{adj} = -0.018$ | | |
| | SS | d.f. | $P$ | SS | d.f. | $P$ | SS | d.f. | $P$ | SS | d.f. | $P$ |
| WW | 0.154 | 1 | *** | 1.437 | 1 | *** | 1.588 | 1 | *** | 0.004 | 1 | 0.811 |
| Sex | 0.223 | 1 | *** | 0.155 | 1 | ** | 0.020 | 1 | 0.614 | 0.065 | 1 | 0.321 |
| Residuals | 0.618 | 57 | | 0.943 | 57 | | 4.329 | 57 | | 3.686 | 57 | |
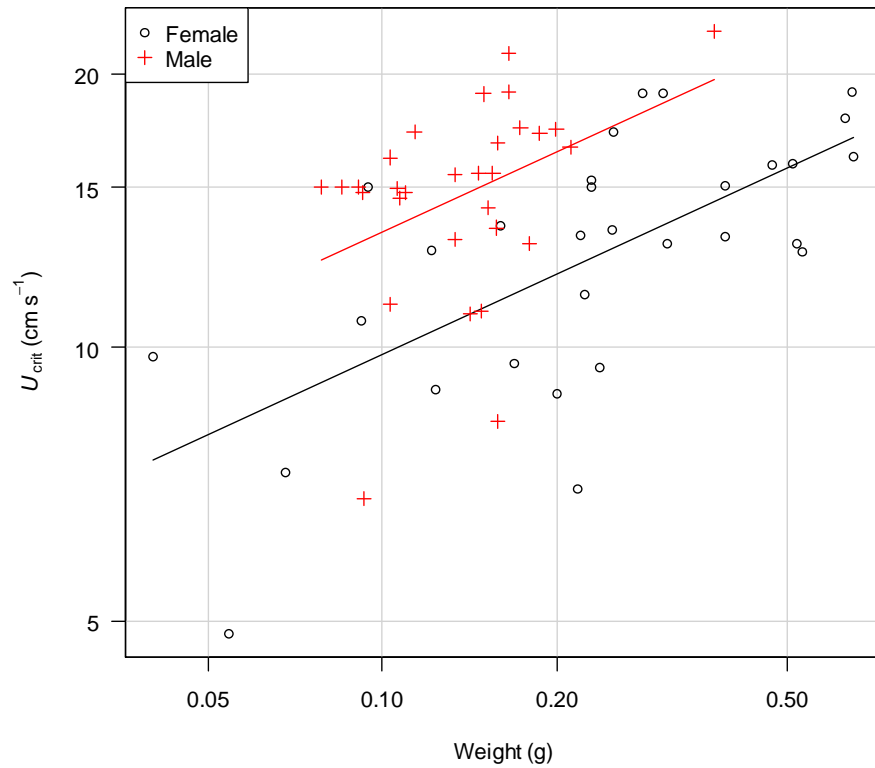


Figure 26: Relationship between critical swimming speed ($U_{crit}$) and mosquitofish weight by sex. Note the log scale of both axes. See Table 9 for regression statistics.
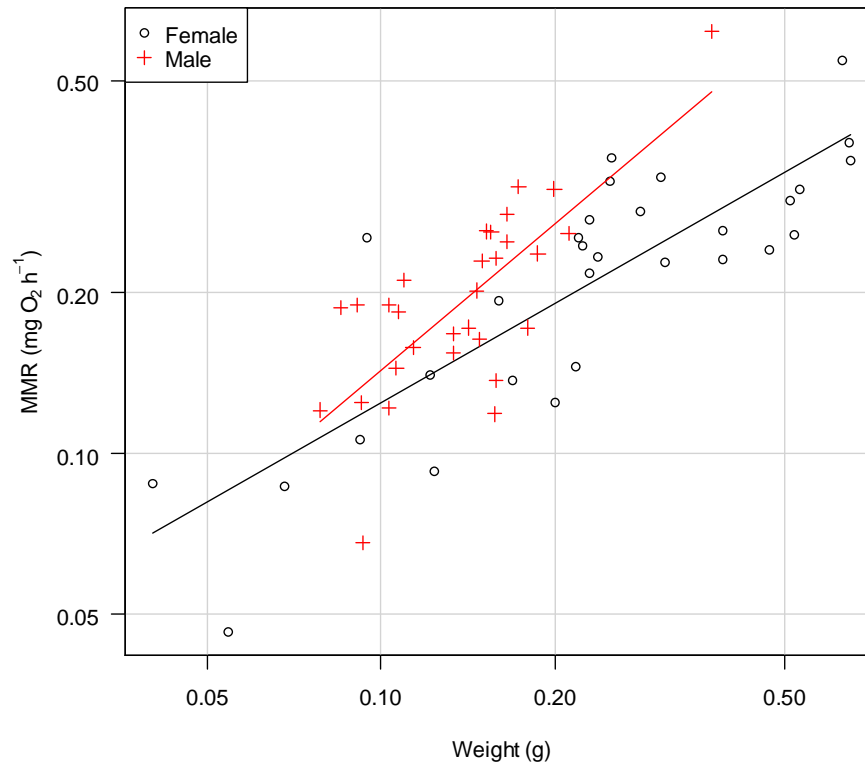
Figure 27: Relationship between maximal metabolic rate (MMR) and mosquitofish weight by sex. Note the log scale of both axes. See Table 9 for regression statistics.

After controlling for fish WW, there was a significant effect of length on MMR (ANCOVA, $P = 0.006$) and differences between sexes (ANCOVA, $P = 0.001$). A multiple regression model showed that MMR was affected independently by weight and length in females but not significantly in males. For the same weight range, larger females had lower MMR values (Fig. 29). Similarly, we found significant effects of MMR on $U_{crit}$ (ANCOVA, $P < 0.0001$), after accounting for fish weight, and differences between sexes (ANCOVA, $P < 0.0001$). This relationship was significant for both males and females, but slightly stronger for the latter ($R^2_{adj} = 0.611$ *vs.* 0.748). $U_{crit}$ mostly depends on MMR, but accounting for this predictor, heavier females had lower $U_{crit}$ (Fig. 30). By contrast, we did not find significant effects of length on $U_{crit}$, RMR or FAS, after accounting for fish weight.
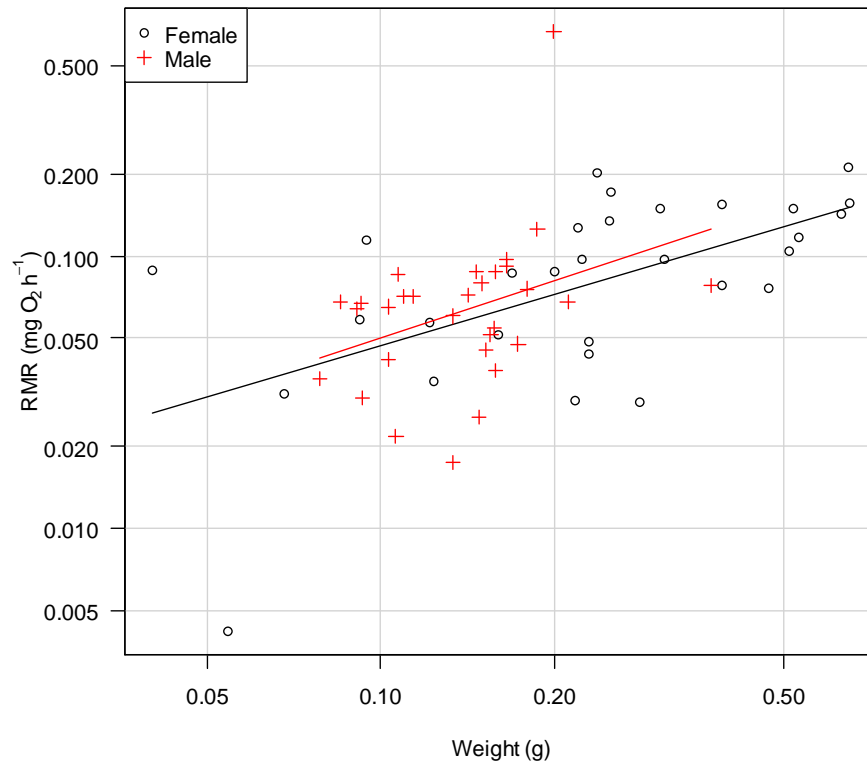
Figure 28: Relationship between resting metabolic rate (RMR) and mosquitofish weight by sex. Note the log scale of both axes. See Table 9 for regression statistics.
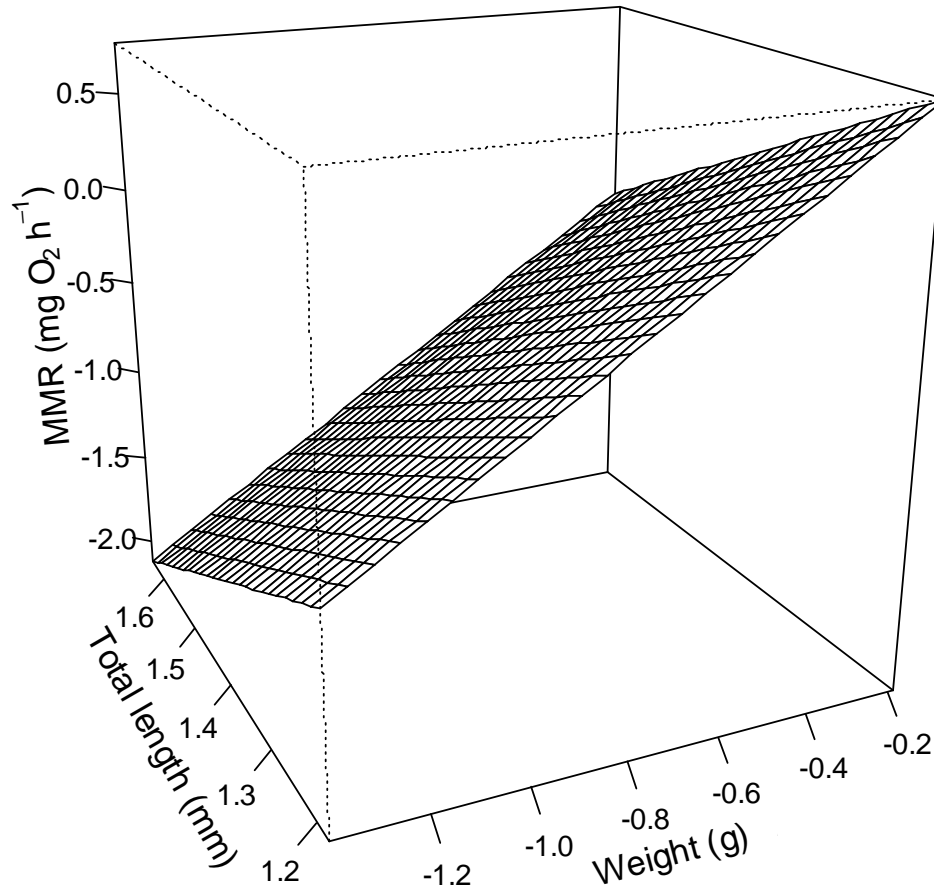
Figure 29: Relationship between maximal metabolic rate (MMR) and weight and total length of female mosquitofish. The three variables are log-transformations and both predictors are significant in a multiple regression model ($R^2_{adj} = 0.752$).
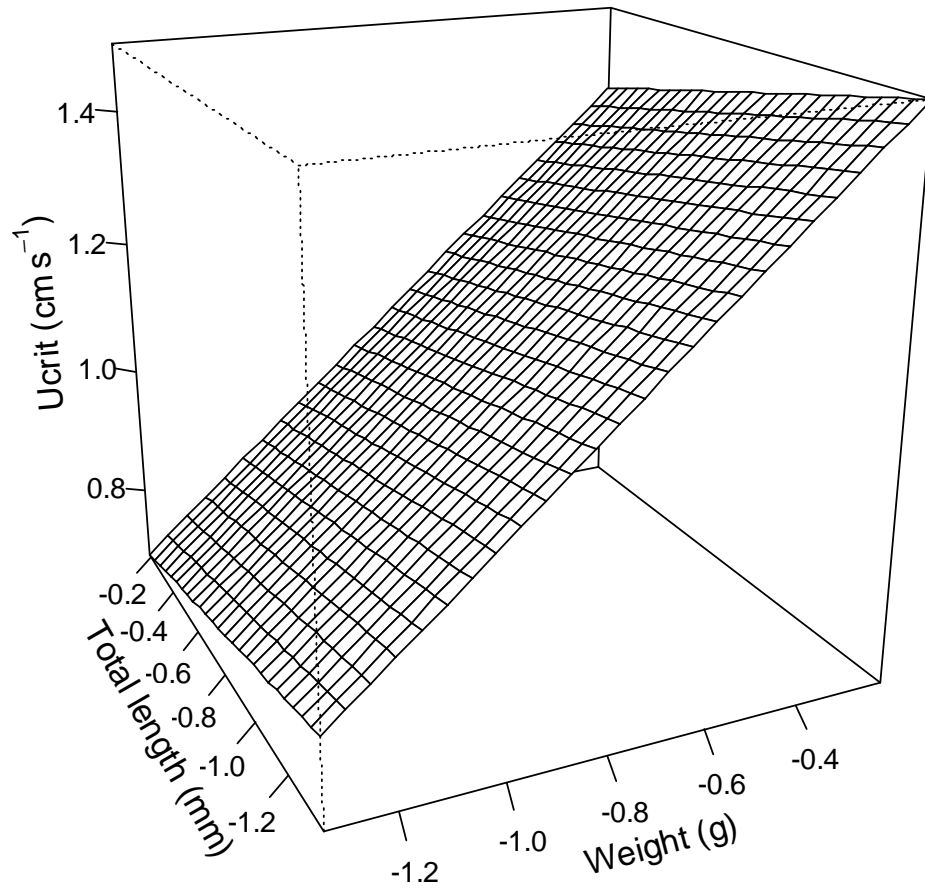
Figure 30: Relationship between critical swimming speed ($U_\text{crit}$) and maximal metabolic rate (MMR) and weight of female mosquitofish. The three variables are log-transformations and both predictors are significant in a multiple regression model ($R^2_\text{adj} = 0.748$).

# Discussion

*Distribution and introduction correlates of mosquitofishes*

We found many errors in the four databases in the number of countries where *G. holbrooki* and *G. affinis* have established and duplicated introduction records in the databases. Differences were more marked for *G. holbrooki* than for *G. affinis*. The most likely cause of these discrepancies are different data sources and taxonomic confusion over the two *Gambusia* species, because before the paper by Wooten *et al*. (1988), they were regarded as two subspecies for many years but since then they have been considered as two valid species. Our new data on the worldwide distribution of mosquitofishes shows that *G. holbrooki* is more widespread and *G. affinis* less widespread than previously described in the literature (e.g. Gerberich 1946; Walton *et al.* 2012) or in databases. Hulme & Weser (2011) have recently demonstrated discrepancies in total numbers of alien species and their establishment between two databases in northern European countries.

The circular statistics confirmed that the two mosquitofish species had significantly different patterns of introduction directions and distances. *G. holbrooki* was introduced predominantly in a northeast direction, whereas *G. affinis* was dispersed towards numerous directions. Introduction distance was longer for *G. affinis* than for *G. holbrooki*. These patterns were relatively uniform throughout the years. *G. holbrooki* was introduced from new introduced areas such as Italy and Georgia to many new neighbouring countries and for these to other countries (Gerberich & Laird 1968; Gerberich 1946). By contrast, *G. affinis* was independently introduced mostly from the native area (USA) and Hawaii to many new countries. Therefore, *G. holbrooki* introduction patterns resemble more a mass dispersal or a jump dispersal (after being introduced to Europe it was introduced directly or indirectly from many countries to many other countries), whereas *G. affinis* resembles more an extreme long-distance dispersal pathway (it was only independently introduced from the USA and Hawaii to many countries) (Wilson *et al*. 2009). These differences between the two species should result in different patterns of genetic structure and enemy release importance (Wilson *et al*. 2009), which deserve future research.

Previous research has demonstrated that socioeconomic variables are important predictors for predicting alien species richness across regions (García-Berthou *et al*. 2005; Hulme & Weser 2011). For example, it has been reported that country area and human

population density are good predictors of alien fish richness in Europe. However, these studies have either been studies on groups of invasive species within Europe or have not considered climatic factors as predictor variables for individual species. In this study we estimated globally what are the most important predictors mediating the RECEIVING COUNTRY and ESTABLISHMENT, ORIGINATING COUNTRY, and INTRODUCTION DATE of *G. holbrooki* and *G. affinis*. In summary, we found climatic, latitudinal and longitudinal factors are more important predictors of RECEIVING COUNTRY and ESTABLISHMENT, whereas country areas, economic and also temperature factors are more important for predicting ORIGINATING COUNTRY and INTRODUCTIONS DATE of mosquitofishes. Detailed discussion of the results follows.

We found that climate and geographical descriptors (latitude and longitude) are the best predictors for RECEIVING COUNTRY (probability of introduction of mosquitofishes), contrary to previous studies on invasive species where country area was more important (García-Berthou *et al.* 2005; Westphal *et al.* 2008; McGeoch *et al.* 2010; Hulme & Weser 2011). García-Berthou *et al.* (2005) found a negative relationship between the number of species introduced to the country and GDPC, but not for the number of species given by a European country. Mosquitofishes have mostly been introduced to countries with warm conditions and less precipitation and frost-day frequency. However, mosquitofishes are mainly introduced for controlling mosquito populations through predation on mosquito larvae (Krumholz 1948). Craig *et al.* (1999) and Bi *et al.* (2003) have demonstrated that malaria transmissions and temperature have a strong, positive correlation. Temperatures $\geq$ 15 $^{\circ}$C are needed for the development of malaria parasites inside mosquitos and climate is very important for predicting malaria incidence (e.g. Craig *et al.* 1999; Gomez-Elipe *et al.* 2007; Mordecai *et al.* 2013). Cold conditions can slow down larval development of Culicid Mosquitoes (e.g. Carrington *et al.* 2013; Ciota *et al.* 2014). Therefore, malaria is a less important issue in cooler countries of higher latitudes, partially explaining the importance of climate and latitudes as predictors of RECEIVING COUNTRY. In general, introduction routes for *G. affinis* are longer than for *G. holbrooki*.

Air temperatures, other climatic variables and latitude are also the best predictors of ESTABLISHMENT of mosquitofishes, although temperatures seem more important for *G. affinis* than for *G. holbrooki* (Fig. 18c, 18d). Mosquitofishes had higher establishment rates under warm temperature conditions, but not for either cold or hot conditions (more extreme

climates). We found unsuccessful establishment in at least 10 out of 61 countries for *G. holbrooki* and 7 out of 57 for *G. affinis*. Mosquitofish are more abundant under warm conditions (Arthington 1989), approximately between 38$^{\circ}$ S and 40$^{\circ}$ N latitude (Krumholz 1944, 1948; Arthington 1991), and have high mortality rates in cold areas during winter (Haynes 1993). The upper lethal water temperature limit is 38 $^{\circ}$C under laboratory conditions (Cherry *et al.* 1976). There was no significant relationship between establishment probability and date of first introduction, in contrast to other fish species (García-Berthou *et al.* 2005).

For ORIGINATING COUNTRY, country area was the best predictor for *G. holbrooki*, whereas exports and GDPC have greater explanatory ability for *G. affinis*. These findings indicate that smaller countries act as ORIGINATING COUNTRY for *G. holbrooki*, whereas countries which have high GDPC and low exports led the donations of *G. affinis*. By contrast, García-Berthou *et al.* (2005) found that large countries acted as ORIGINATING COUNTRY for invasive fish species within European countries. Hulme & Weser (2011) found differences between databases for richness of alien species per country in 13 northern European countries, the country area being more important in the North European and Baltic Network on Invasive Alien Species database while human population density has greater explanatory ability in the Delivering Alien Invasive Species Inventories for Europe database. They also found some significant effects of GDPC for a few taxonomic groups (e.g. phytoplankton), but not for fish, in contrast to Leprieur *et al.* (2008). Similarly, Westphal *et al.* (2008) concluded that the degree of international trade (e.g. merchandise imports) and country area were the best predictors of alien invasive species richness at the global scale. In other studies, country area and human development index were the most important predictors of richness of alien invasive species at the global scale (Dalmazzone 2000; Westphal *et al.* 2008; McGeoch *et al.* 2010).

For INTRODUCTION DATE, we found minimum and mean temperatures were the most important predictor variables for both species, whereas country area was the best for *G. affinis*. These findings suggest that both species have been more recently introduced to warmer countries (Fig. 12 & 13), but *G. affinis* has been more recently introduced to smaller countries (e.g. Pacific islands; Fig. 13). It is to be expected that large countries will have earlier introductions for a number of reasons often rich economies and more diverse temperatures that might enhance the probability of establishment.

*The ecological impacts of mosquitofishes*

The meta-analysis confirmed that invasive mosquitofishes have important ecological effects on a range of aquatic biota, notably fish, macroinvertebrates and amphibians. The mean effects were stronger for fish than for macroinvertebrates and amphibians. Impacts of mosquitofishes on native fish are well known, as they reduce growth and survival rates through predation, resource competition, and aggression (e.g. Rincón *et al*. 2002; Mills *et al*. 2004; Caiola & de Sostoa 2005; Rowe *et al*. 2007; Thompson *et al*. 2012). Effect sizes varied among fish families, probably related to similarity in fish sizes and ecological niches; for instance, small-sized fish (e.g. Cyprinodontidae, Galaxiidae and Poeciliidae) sustained more negative mean effects than large-sized ones (e.g. Cyprinidae, Melanotaeniidae and Ictaluridae). The dependency of mosquitofish effects on fish size is well known (Taylor *et al*. 2001; Goldsworthy & Bettoli 2006; Henkanaththegedara & Stockwell 2013). Among amphibians, Urodela (newts and salamanders) received more harmful effects than Anura species (frogs and toads), because some species of toads (*Bufo marinus*, *B. melanostictus*) and frogs (*Crinia georgiana*, *Litoria moorei*, *L. adelaidensis*, *Lithobates clamitans*) were not affected (Komak & Crossland 2000; Reynolds 2009; Karraker *et al*. 2010; Shulse *et al*. 2013), and these effects vary with prey availability and species-specific habitat use (Lawler *et al*. 1999; Preston *et al*. 2012; Shulse *et al*. 2012). In general, tadpole survival, rate of development, or size at metamorphosis has been shown to be lower in mosquitofish presence than in controls (e.g. Lane & Mahony 2002; Baber & Babbitt 2003; Segev *et al*. 2009; Karraker *et al*. 2010; Smith & Dibble 2012).

Impacts at lower trophic levels are more variable. Mosquitofish predatory effects on Diptera and Crustaceans are already known (e.g. Hurlbert & Mulla 1981; Miura *et al*. 1984; Clem & Whitaker Jr. 1995; García-Berthou 1999) but depend on alternative prey (Blaustein 1992). Our results agree with previous literature that has shown that effects on Mollusca and other insects (i.e. damselflies, dragonflies, beetles, and giant water bugs) were less clear (Miura *et al*. 1984; Cardona 2006; Shulse *et al*. 2013). Similarly, although there is no significant overall effect for zooplankton, the results are significant for Copepoda (Cyclopoida and Calanoida) and Cladocera, which decline in density and biomass after introduction of mosquitofish (e.g. Soto & Hurlbert 1991; Cabral *et al*. 1998; Leyse *et al*. 2004; Ning *et al*. 2010). Rotifera also increased in general in presence of mosquitofish, due to the reduction of large competitors (cladocerans, calanoid and

copepods) and predators (cyclopoid copepods) via mosquitofish predation (Miracle et al. 2007).

Although mosquitofish can consume algae and plants (Clem & Whitaker Jr. 1995; García-Berthou 1999; Blanco et al. 2004; Gkenas et al. 2012), our results show no overall effects on Periphyton and bacteria. Furthermore, ecosystem effects of mosquitofish were not consistent. Therefore, although mosquitofish have clear effects on higher trophic levels, these are much more variable at lower trophic levels and often do not cascade to the whole ecosystem (Cardona 2006).

The results of the meta-regression agree with previous studies, in which higher mosquitofish density or temperature yield greater effects (Taylor et al. 2001; Mills et al. 2004; Rowe et al. 2007; Thompson et al. 2012; Carmona-Catot et al. 2013). Similarly, the effects increase with experimental duration (see e.g. Lawler et al. 1999; Ling & Willis 2005; Segev et al. 2009; Karraker et al. 2010; Akhurst et al. 2012) but decrease with water depth of experimental setting (Ling & Willis 2005).

Our meta-analysis revealed strong differences among variable types with general increases in aggressive acts received in the presence of mosquitofish and reductions in density/biomass in a range of taxa such as fish, amphibians, macroinvertebrates, and zooplankton. Many experimental studies have demonstrated that mosquitofish have strong agonistic interactions with coexisting species such as fish (e.g. Rincón et al. 2002; Laha & Mattingly 2007; Rowe et al. 2007; Keller & Brown 2008; Priddis et al. 2009) and amphibians (e.g. Gamradt & Kats 1996; Morgan & Buttemer 1996; Baber & Babbitt 2003; Segev et al. 2009; Preston et al. 2012). Native fish and amphibians reduce their foraging activities and increase refuge use when mosquitofish were present (e.g. Lawler et al. 1999; Rincón et al. 2002; Becker et al. 2005; Gregoire & Gunzburger 2008; Smith et al. 2011). Moreover, mosquitofish reduce prey availability and predation rates of native fish (Belk & Lydeard 1994; Gamradt & Kats 1996; Ling & Willis 2005; Thompson et al. 2012; Henkanaththegedara & Stockwell 2013) and sharply reduce microinvertebrate abundance (Soto & Hurlbert 1991; Hansson & Carpenter 1993; Cabral et al. 1998; García-Berthou 1999; Leyse et al. 2004).

By contrast, effects on other variable types (i.e. size/stage, life history, diversity, feeding behaviour and other behaviour) were much less consistent. Native fish may change their life-history to reduce the impact of introduced competitors but these changes take

place in the longer term, while their abundance and biomass is reduced more rapidly (Olden *et al.* 2006; Strauss *et al.* 2006). For invertebrates, for example, mosquitofish induce enhanced fecundity, offspring per female and brood number of *Daphnia longispina*, but body length is often not affected (Castro *et al.* 2007). However, fish effects on life-history traits of cladocerans is complicated due to its moderation with additional factors, such as food level (Reede 1997; Weber & Van Noordwijk 2002) and temperature (Sakwinska 1998). In amphibians, some species (e.g. *Limnodynastes tasmaniensis*, *C. signifera*) show no effects on time to metamorphosis, size of the froglets or feeding activity (Lane & Mahony 2002) although other frog species (e.g. *Rana aurora draytonii*) showed harmful effects on size at metamorphosis (Lawler *et al.* 1999). Preston *et al.* (2012) found that western toads (*Anaxyrus boreas*) are larger and metamorphosed more quickly in the presence of mosquitofish, and suggesting that mosquitofish reduced inter-specific competitors (i.e. tree frog tadpoles).

In conclusion, our analyses have demonstrated that invasive mosquitofishes have important ecological effects on a range of aquatic biota, particularly fish, macroinvertebrates and amphibians. Although mosquitofishes have strong agonistic behaviour and sharply reduce density and biomass of native species, the effects vary markedly among taxonomic groups and variable types and are context-dependent.

*Swimming capacity and metabolism of eastern mosquitofish*

Our results on the experiment to analyse the swimming capacity and metabolism of eastern mosquitofish partially supported our hypothesis from the variation of swimming speed and metabolic rates with size. In particular for $U_{crit}$ and MMR, the relationship was positive and better fitted with fish weight, a proxy of size in fish. Body weight may be a better correlate to speed and maximum metabolism because of this biological trait is directly related to the body volume and consequently, to swimming thrust and energy expenditure against the current (Boisclair & Tang 1993; Post & Lee 1996; Ohlberger *et al.* 2005). Plaut (2000) showed a linear positive relationship of $U_{crit}$ with fish length in female *G. affinis*. As far as we know, our study is the first to directly compare the critical swimming speed of male and female mosquitofish. We observed that males had higher $U_{crit}$, than females, after controlling for fish size. By contrast, in a very-well studied poeciliid, the guppy *Poecilia reticulata*, males preferred microhabitats with lower velocities than females both in experimental channels (Hockley *et al.* 2013) and in the wild (Magellan & Magurran 2006).

The sex differences in metabolic rates and swimming ability that we observed in mosquitofish might be related to the sexual dimorphism of mosquitofish. Female mosquitofish have larger fins, which significantly increase the hydrodynamic drag under high flow levels (Nicoletto 1991). In case of Mateus *et al*. (2008)'s study, these authors analysed the Iberian barbel *Luciobarbus bocagei* (Steindachner 1864), a non-sexually dimorphic species, with similar body shape and consequently, hydrodynamics between sexes. Also, cyprinid females are oviparous and thus, reproductive investment is moderate in relation to poeciliid females, making swimming performance more similar between sexes. Regarding RMR, we did not find differences between sexes. This suggest that metabolism only shows sex-dependent differences after prolonged physical exercise, whereas out of flow conditions oxygen consumption should be changed for both sexes. In more detail, females may have a higher metabolic expenditure due to physiological reproductive investment (see comments above), but males may invest more in locomotion through searching for breeding partners or displaying territorial behaviours (Kolok 1999), thus compensating for potential sexual differences at the resting level.

FAS did not vary significantly with size or sex. This indicates that MMR and RMR similarly increased with size (i.e. the same regression slope), resulting in a flat slope for the linear function for FAS. Although a large amount of evidence demonstrates the high dependence of FAS on size in fishes (Killen *et al.* 2007), similar *b* coefficients between MMR and RMR have been also observed, with this ratio remaining virtually constant with increase in weight (e.g. Huang *et al.* 2013). The invariant FAS of mosquitofish suggests that aerobic capacity might not increase as their bodies grow, which is consistent with its lower locomotor performance with respect to other species (see Table 10 and more examples in Yan *et al*. 2012). However, the lack of sexual differences for FAS was puzzling, as males showed higher MMR levels, whereas RMR was similar between sexes. Therefore, FAS should have been higher in males than females. Again, a small sample size and the high data dispersion across sexes may 'dilute' sexual differences for this metabolic ratio.

The present study also shows multiple interactions of involved factors, both independent and response variables to modulate swimming performance and metabolism traits. This is probably related to a wide inter-individual variation (Kolok 1999), being highly associated with Darwinian fitness (Plaut 2001). Specifically, MMR was lower in larger fish for the same weight, suggesting a 'slender' body shape and consequently, a

lower hydrodynamic drag (Vogel 1994). Besides this, 'heavier' fish reached reduced speed within the same MMR range, probably due to a lower available energy level for propulsion of a bigger body mass (Ohlberger *et al.* 2005). These findings were more clearly observed in females, which certainly display higher variability of body shapes and wider weight range (Plaut 2002).

These results show that swimming speed and associated oxygen consumption are highly variable factors. Thus, this is not only a matter of morphology and hydrodynamics, but also involves species- and individual-specific metabolism traits (Plaut 2001), with pivotal relevance of the particular trophic niche (i.e. prey, mid-predator, top-predator) occupied in the aquatic food web (e.g. Fu *et al.* 2009). According to the data shown in Table 11, eastern mosquitofish appear to be the least adapted to flowing-waters (i.e. narrow range of 'flow niche'), even within the same taxonomic family (Poeciliidae) or genus (*Gambusia*), with a mean $U_{\mathrm{crit}}$ <15 cm s$^{-1}$. Consequently, its high invasive rate across the world (including rivers and streams) may be more related to behavioural adaptations (e.g. active search for refuges) rather than swimming capacity, as suggested by Ward *et al.* (2003) for several non-native fishes. Also, its particular reproductive strategy (i.e. livebearing) provides this species with a great capacity to re-colonize sites after high flow conditions, compensating for the low swimming capacity (Chapman & Warburton 2006).

Table 11: Compilation of critical swimming speeds ($U_{crit}$) in a variety of small fishes. Fork length (FL), standard length (SL) and total length (TL) are shown as measures of body size. *Kolok & Oris (1995) only reported wet weight for *Pimephales promelas*, such that the current SL range for this fish species was calculated from data shown in Godard *et al.* (2013).

| Species (taxonomic family) | Size | Range/Mean ± SE (mm) | $U_{crit}$ | Variation statistic | Variation values | References |
|---|---|---|---|---|---|---|
| *Gambusia holbrooki* (Poeciliidae) | TL | 15–44 | 14.11 cm s$^{-1}$ | Range | 4.85–22.26 | this study |
| *Gambusia holbrooki* (Poeciliidae) | TL | 16.17 ± 0.02 | 14.37 cm s$^{-1}$ | SE | 3.23 | Seebacher *et al.* (2012) |
| *Gambusia affinis* (Poeciliidae) | SL | 28–35 | 25.00 cm s$^{-1}$ | SE | 1.30 | Plaut (2002) |
| *Gambusia affinis* (Poeciliidae) | TL | 38–45 | 38.54 cm s$^{-1}$ | 95% CI | 2.07 | Ward *et al.* (2003) |
| *Poecilia reticulata* (Poeciliidae) | SL | 17.60 ± 0.04 | 22.60 cm s$^{-1}$ | SE | 0.79 | Nicoletto (1991) |
| *Cyprinodon pecosensis* (Cyprinodontidae) | SL | 27–38 | 36.28 cm s$^{-1}$ | SE | 1.16 | Kodric-Brown & Nicoletto (1993) |
| *Hypomesus transpacificus* (Osmeridae) | SL | 35–53 | 28.00 cm s$^{-1}$ | SE | 5.00 | Swanson *et al.* (1998) |
| *Pogonichthys macrolepidotus* (Cyprinidae) | SL | 20–30 | 30.77 cm s$^{-1}$ | SE | 2.68 | Young & Cech Jr. (1996) |
| *Pimephales promelas* (Cyprinidae) | SL | 35–45 | 35.90 cm s$^{-1}$ | Range | 28.80–43.40 | Kolok & Oris (1995)* |
| *Fundulus heteroclitus* (Fundulidae) | TL | 70.36 ± 0.94 | 6.99 SL s$^{-1}$ | SD | 1.27 | Yetsko & Sancho (2015) |
| *Fundulus majalis* (Fundulidae ) | TL | 70.23 ± 0.97 | 8.64 SL s$^{-1}$ | SD | 1.70 | Yetsko & Sancho (2015) |

*Management implications*

We have identified clear worldwide introduction pathways and contrasting histories for eastern and western mosquitofish that have management implications. Prevention of new introductions is the most effective management tool in invasion biology (e.g. Ricciardi & Rasmussen 1998; García-Berthou *et al.* 2005; Schlaepfer *et al.* 2011). We have identified some countries or regions (e.g. Italy, Hawaii, Georgia, South Africa) that acted as donors of introductions to many countries and should receive more attention in their trade, fisheries, and transport regulations. The routes identified might be the same for future invaders, if these socioeconomic vectors have not changed in recent years.

Context-dependency is well appreciated in invasion biology (Alcaraz *et al.* 2008; Blanchet *et al.* 2009; Pyšek *et al.* 2012). In freshwater ecosystems, abiotic factors (e.g. water quality, substrate composition and flow regime) have been considered as important predictors of ecosystem invasibility (Lapointe *et al.* 2012; Marchetti *et al.* 2004; Murphy *et al*. 2015). We have shown that temperature and climate in general (Study 1) and water flow (Study 3) influence the invasive success of mosquitofish. Mosquitofish is a small-bodied fish, inhabiting the water-column and surface microhabitats. Their swimming ability is low compared to other small fish species (see Table 11). These traits suggest that water management may help to control the spread of mosquitofish throughout Iberian fresh waters and elsewhere. Some measurements can be suggested according to the present findings. For example, a particular high flow level (i.e. water velocities $>20$ cm s$^{-1}$) may be maintained nearby still-waters where mosquitofish is present to control or possibly erradicate their populations and prevent future spread. In regulated rivers with mosquitofish, dams could release water, whereas abstraction should be reduced during the breeding period (i.e. late spring) to allow a higher water flow. These management actions should particularly affect females, which would be dragged downstream due to a lower swimming capacity. Females should also expect to spend a large amount of energy to withstand the current and remain in the area, thus decreasing the overall reproductive rate of mosquitofish population in the regulated reaches. Furthermore, natural flooding events should be allowed, as are typical in Mediterranean-climate rivers during autumn-winter (Gasith & Resh 1999). Thus, native species might recover their 'flow niche' and prevent re-colonization of invasive fishes (Ward *et al.* 2003). Water resource development and non-native species have been demonstrated as primary drivers for the decline of native fishes in lotic habitat types, and decreasing water flows in rivers and streams due to dam

construction and water extraction can provide suitability for new invasions of mosquitofish (Gibson *et al.* 2014; Pool & Olden 2014). Maintenance of high flow regimes is beneficial to native fishes in lotic habitats, and high-flow events may play as a mechanical removal of non-native fishes, particularly during reproductive periods of non-native species within the system (Propst & Gido 2004; Gido *et al.* 2013; Pool & Olden 2014). Water flow experiments are important for freshwater ecosystems and management (Olden *et al.* 2014). These results may contribute to biological conservation, restoration efforts and sustainable freshwater management for stakeholders, managers and policy makers.

# General Conclusions

1) We have shown clear errors in four widely used internet databases and the literature regarding the current distribution and introduction history of two of the most widely distributed invasive fish, *Gambusia holbrooki* and *G. affinis*.

2) We estimate that *G. holbrooki* has established in ca. 49 countries (a number much higher than previously considered) and *G. affinis* in 44. These two poeciliid fishes have established in all continents except Antarctica but *G. holbrooki* is mainly present in southern Europe, the Middle East, northern and western Africa, western Asia and Australia, whereas *G. affinis* is present mainly in the Americas, southern Africa and eastern Asia.

3) Although the total number of introductions of the two species is similar, *G. holbrooki* was mostly introduced to the wild earlier (on average) with a peak of intensity in the 1920s, whereas the introductions of *G. affinis* are dominant in the second half of the XXth century and constant through time. There is no significant relationship between establishment rates and date of first introduction to the country.

4) Climate (e.g. frost-day frequency, precipitation, minimum and mean temperatures), latitude and longitude are good predictors of introduction and establishment probabilities of mosquitofish, whereas country area, socioeconomic factors (e.g. gross domestic product (GDP) per capita, % exports of GDP), and minimum temperature were better at predicting number of donations to other countries and date of introduction.

5) A meta-analysis of the literature shows similar ecological impacts for the two mosquitofish species.

6) The impacts of these two species are clear and strong for fish, macroinvertebrates, amphibians, and some zooplanktonic groups (such as copepods and rotifers) and more variable for taxa at lower trophic levels and for ecosystem features.

7) The ecological impacts of mosquitofishes are general for the number of aggressive acts received by native species and for decreases in density and biomass but more variable for other response variables such as size structure, life history traits, or other behavioural traits.

8) The ecological effects are heterogeneous and context-dependent on a number of features such as the target species involved, the experimental setting (depth of the study system, density of fish used), and environmental factors such as temperature.

9) We estimated the mean critical swimming speed of *G. holbrooki* as 14.11 cm s$^{-1}$ (range = 4.85–22.26), which is lower than for many other fish of similar size and confirms that this species is limnophilic and its invasive success might be partially explained by hydrological alteration.

10) The critical swimming speed and maximal metabolic rate vary markedly with fish size and sex, with males having much higher values for the same weight, and thus being more resistant to strong water flows.

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# Appendix

List of primary studies that fit the inclusion criteria and were included in the meta-analysis.

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