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*Gambusia holbrooki*: implications for management and control**

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# Daily ageing to delineate population dynamics of the invasive fish *Gambusia holbrooki*: implications for management and control

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## Abstract

Despite the high global invasion profile of *Gambusia holbrooki*, there is limited knowledge of its population dynamics, especially biological parameters for fine-scale delineation of recruitment events that can provide a basis for controlling this short-lived invasive species. Key to this is daily ageing of otoliths, and in this study 193 (~50%) of 394 wild fish sampled from an invaded wetland were successfully aged. Increment counts of growth rings in otoliths from aquaria-reared *G. holbrooki* did not correspond 1:1 to their known ages but were significantly correlated ( $P < 0.05$ ,  $R^2 = 0.92$ ), suggesting the use of linear relationships could nevertheless be used to estimate the age of wild-caught fish. Mean daily growth rate estimated for spring-summer cohorts (0.28 mm/day) was much greater than that of winter cohorts (0.12 mm/day). A natural mortality rate of  $0.01 \text{ day}^{-1}$  for females was estimated by catch-curve analysis via length to age conversion based on the growth relationship. Birth rate closely correlated with changes in water temperature with a peak reproduction in January (Austral summer) at the study site. This study, for the first time, used daily age estimates of *G. holbrooki* to quantitatively examine population dynamics, which can be used to investigate invasive dynamics and choice of pest control strategies, including their scheduling and assessment.

## INTRODUCTION

The introduction and establishment of alien plants, animals, and microbes, commonly known as biological invasions, threaten native fauna and environments (Srean 2015). Biological invasions cause losses in economic and environmental values, particularly in freshwater ecosystems (Pimentel et al. 2001; Ricciardi and Rasmussen 1998). For example, in Australia, the combined cost (financial losses and control costs) of invasive pest species was estimated to be approximately \$13.6 billion in the 2011/12 fiscal year, which is equivalent to 0.29% of GDP (Hoffmann and Broadhurst 2016).

Native to the United States of America and Mexico, *Gambusia affinis* and *G. holbrooki* had been deliberately introduced globally as mosquito-control agents, with the latter species introduced to Australia (Arthington and Lloyd 1989) and Tasmania (Keane and Neira 2004; Lynch 2008). However, *Gambusia* sp. frequently compete with native species for habitat and food resources as well as actively attacking them and predating on their eggs and larvae (Pyke 2008). For instance, in Australia, *G. holbrooki* consumes *Limnodynastes ornatus* larvae (Komak and Crossland 2000); threatens the vulnerable native ornate rainbowfish *Rhdinocentrus ornatus* (Keller and Brown 2008); and nips the tails of *Crinia signifera* (common froglet), *Lthmodynastes dumerilii insularis* (pobblebonk) and *Litoria ewingii* (brown tree frog) tadpoles (Lynch 2008). Most recently, *G. holbrooki* was responsible for the localised extinction of a critically endangered fish, *Scaturiginichthys vermeilipinnis*, from 30 of its 37 last remaining artesian-spring habitats (Nicol et al. 2015) and was responsible for the decline of at least nine other native fish populations (DPI 2018). Globally, *Gambusia* sp. are listed among the top 100 pest species (Lowe et al. 2000).

To develop effective control strategies, it is necessary to understand the population dynamics of target pest populations and estimate their incursion rates. In practice, these approaches centre on examining the age-structures of the populations to reveal crucial information about their birth (i.e., recruitment events), mortality, growth, and other essential biological parameters (Campana and Jones 1992; King 2007). Firstly, knowledge of mortality, recruitment and growth rates and their dynamics (e.g., recruitment frequency and window) can assist to make informed decisions on the choice of control measures (e.g., reproductive sabotage and or physical removal) and the time of their deployment (Brown and Walker 2004; Laplanche et al. 2018; Taylor et al. 2012). Secondly, these are essential to parametrise and assess genetic pest control strategies such as the Trojan Y (Gutierrez et al. 2012; Gutierrez and Teem 2006) and monitoring their efficacy post implementation.

Several studies have used scales to determine the annual age of *Gambusia* sp. (Cabral and Marques 1999; Carmona Catot 2013; Erguden 2013; Patimar et al. 2011; Vargas and de Sostoa 1996). However, the use of yearly age-classes is largely inappropriate for short-lived species such as *Gambusia* sp., where almost all critical life processes occur within an annual cycle. Typically, *Gambusia* sp. populations also undergo seasonal (i.e., intra-annual) boom and bust cycles in invaded habitats (Lynch 2008; Pyke 2005; Sloterdijk et al. 2015), making yearly ageing all the more inadequate. Hence, there is a need for ageing information on a finer than annual scale for more accurate quantification of recruitment, mortality, and the intra-annual dynamics of the

incursions that can be exploited for control strategies. For example, the delineation of recruitment events and their prediction could allow management interventions that are targeted, cost-effective and for assessing their effectiveness (Diggle et al. 2012; Taylor et al. 2012) among others.

In fish, annuli (i.e., "age rings") in hard parts, deposited in response to circadian periodicity, have been successfully used to determine age (in days) of juveniles of commercial fish species (Cerna and Plaza 2016; Dougherty 2008; Kupchik and Shaw 2016). Despite this, to our knowledge, this has never been explored for *G. holbrooki*. In this context, our study aimed to test whether rings in otoliths could be utilized to determine daily age (hereafter referred to as ageing) of *G. holbrooki* to estimate vital population parameters – length at age, birth rate and daily growth rate, mortality – to reveal insights that can be applied in controlling this notorious pest fish.

## **MATERIALS AND METHODS**

### **Fish sampling**

*Gambusia holbrooki* were sampled monthly (March to June 2018) by scoop netting (2 mm mesh-size) from an infested estuarine wetland near Tamar Island Wetland Reserve (TIWR), Tasmania, Australia. Gravid females were also held in aquaria for breeding, thereby supplying a sample of individuals of known age. The term "known age" with the unit of day post birth (dpb) will be hereafter used to refer to the number of days post birth. For ageing, 394 wild and 174 reared (at  $21 \pm 1$  °C and 14:10 h light-dark cycle) fish were sexed, measured, and weighed before undertaking otolith removal and preparation. Of the 174 reared fish, 14 were from an earlier year-class, but of known age.

Fish were randomly selected, then euthanized using AQUI-S. Total length (TL) was measured to the closest 0.1 mm. Males were distinguished by the presence of a gonopodium and lack of a gravid spot on their abdomen, and the opposite of these criteria was used for mature females. The sex of each fish was then categorized into one of three groups: (M) mature male, (F) mature female, and (I) indeterminate. Water temperature in the wild was measured using a Wp-81 pH/Temperature Meter.

A weekly capture dataset corresponding to the study period (2017–2018) was also extracted from a long-term time-series of population monitoring data at the TIWR (data courtesy J. Duggin, TIWR). Briefly, this consists of capture data obtained using standardized effort once weekly.

### **Ageing procedure**

Sagittae (hereafter referred to as otoliths) were removed, air-dried, and mounted on labelled slides using Super Glue (Cyanoacrylate Glue). A set of 1500, 2000, 2500 grit sandpapers, and a set of 9, 5, 1 µm polishing films were used to grind and polish (one side) mounted otoliths. The progress of polishing was assessed under a compound microscope with transmitted light.

A compound microscope fitted with a built-in digital camera was used to examine otolith microstructure. Growth increment counts (for both known-age and wild fish) were undertaken blind, i.e., without the readers having prior knowledge of the actual age, size, or origin (wild/reared) of the samples.

## **Data analysis**

Sex ratio (F: M: I) was computed on an annual and monthly basis.

### **A) Validation and estimation of fish age:**

Of the 174 aquarium-reared fish, 62 otoliths (fish) were utilized to validate the age determination of *G. holbrooki*. The remaining samples could not be used due to either an inability to retrieve the otolith, damage sustained during polishing, or poor readability. Preliminary age examination revealed that the number of growth increments did not exactly equal known age, thereby suggesting that the use of linear regression would be necessary to estimate the age of wild fish (estimated age hereafter referred to as age). Each sample was examined twice independently by two readers, and the average percent error (APE) was calculated. If  $APE \geq 10\%$ , increment counts were excluded from analyses (ICES 2014).

### **B) Growth modelling and mortality:**

The von Bertalanffy growth model (VBGM) (von Bertalanffy 1957) was applied to address the TL-at-age data of wild *G. holbrooki* in this study.

$$L_t = L_\infty - (L_\infty - L_0)e^{-Kt} \quad (1)$$

Where

$L_t$  is the TL at age  $t$  (mm),  $L_\infty$  is the asymptotic TL (mm) and  $t$  is the age (day)

$L_0$  is the hypothetical TL at day zero of age (mm)

$K$  is the growth coefficient ( $\text{day}^{-1}$ )

Male and female data were analysed separately, as it was clear that growth was sexually dimorphic.

However, to avoid the omission of young age classes, the immature fish data was included into

female and male datasets for modelling. This was based upon the presumption of an equal sex ratio of *G. holbrooki* at birth (Haynes and Cashner 1995).

C) VBGM parameters ( $K$ ,  $L_{\infty}$ ,  $L_0$ ) were applied to generate age-length keys for female and male *G. holbrooki* to compute the mortality rate ( $M$ ) using catch curve analysis (Beverton 1957).

D) Birth rate and growth rate calculation:

Date of birth was back-calculated using the age of wild fish, and frequency distributions of monthly birthed fish were examined in comparison with changes in water temperature. Frequency distributions of mature females with no, small, and large gravid spots were also generated for the time of sampling (monthly) to support the back-calculation birth rate. Mean counts of fish captured per scoop were plotted versus time and compared with monthly birth rates. Besides, correlation analysis (Pearson method) was performed to examine if there is a significant relationship between birth rate and temperature.

Total length at age was used to estimate the daily growth rate. Frequency distributions of growth rate were plotted, and a one-way ANOVA was used to test whether there were significant differences in growth rate among fish birthed at different times of the year.

## RESULTS

Of the 394 wild fish processed, 193 (~50%) fish were successfully aged. The other ~50% of 394 fish were discarded from the data analysis due to otolith losses and damaged samples ( $n = 183$ , collectively), or  $APE \geq 10\%$  ( $n = 18$ ).

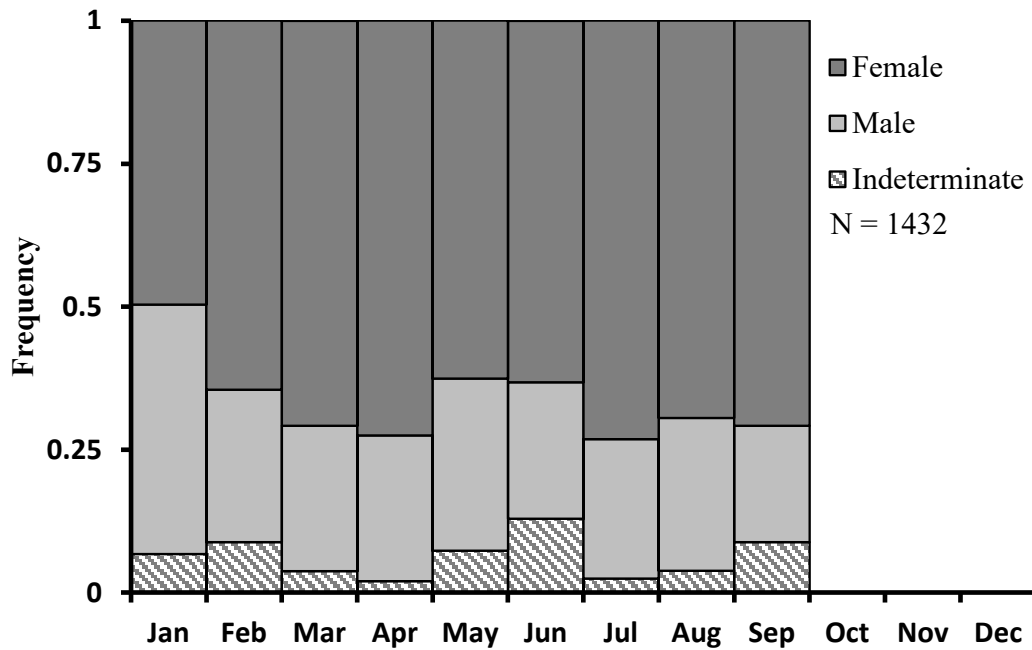


Fig. 1 Sex ratio of *G. holbrooki* between January and September 2018

The female/male ratio was 2.3/1.0 on average, and although the sex ratio of wild-caught fish varied between months, mature females outnumbered males and immature individuals throughout the study period (Fig.1).

#### A) Ageing and age estimates:

Typically, the sagittae had an ovoid appearance, tapering downwards anteriorly, with a conspicuous concave depression along the ventral hind margin (Fig.2A). Post-polishing, growth increments appeared as a combination of an opaque and a translucent increment when viewed under a compound microscope with transmitted light (Fig.2B).

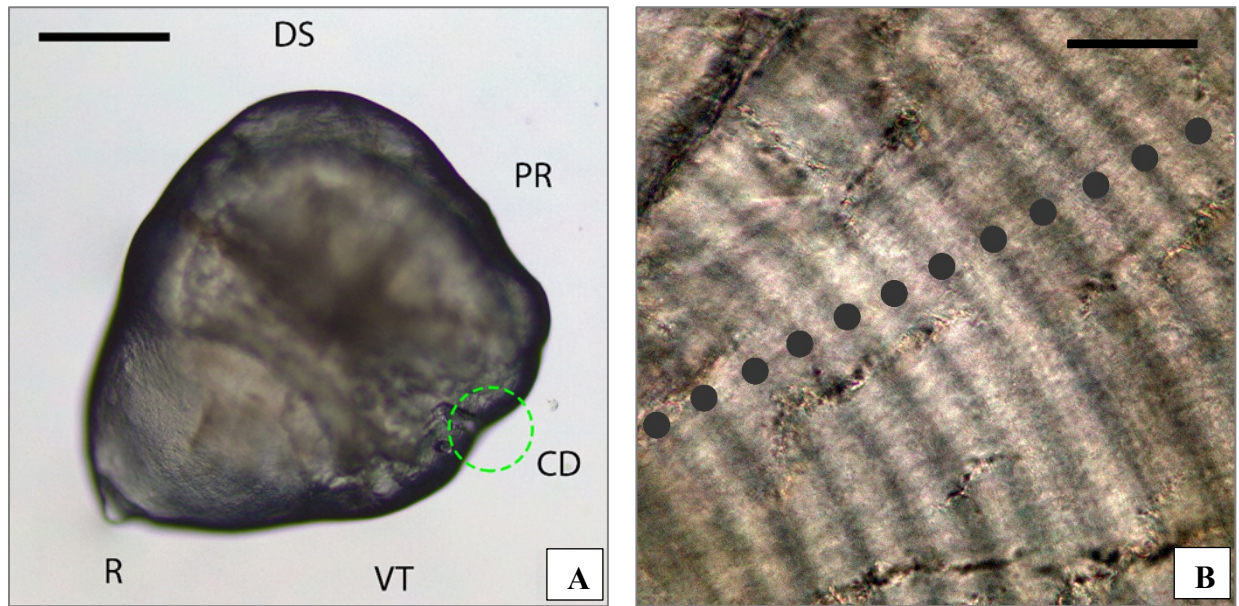


Fig 2. (A) a sagitta extracted from a mature female (age=126 days old), showing relative orientation in the fish. DS: dorsal, VT: ventral, R: rostrum, PR: post-rostrum, and CD: concave depression (dotted circle) on ventral margin of the sagitta was a common feature to all sagittae. Scale bar: 0.2 mm; (B) increments as observed in a polished sagitta of a wild fish (TL=19 mm, estimated age=38 dpb). Scale bar = 20  $\mu$ m.

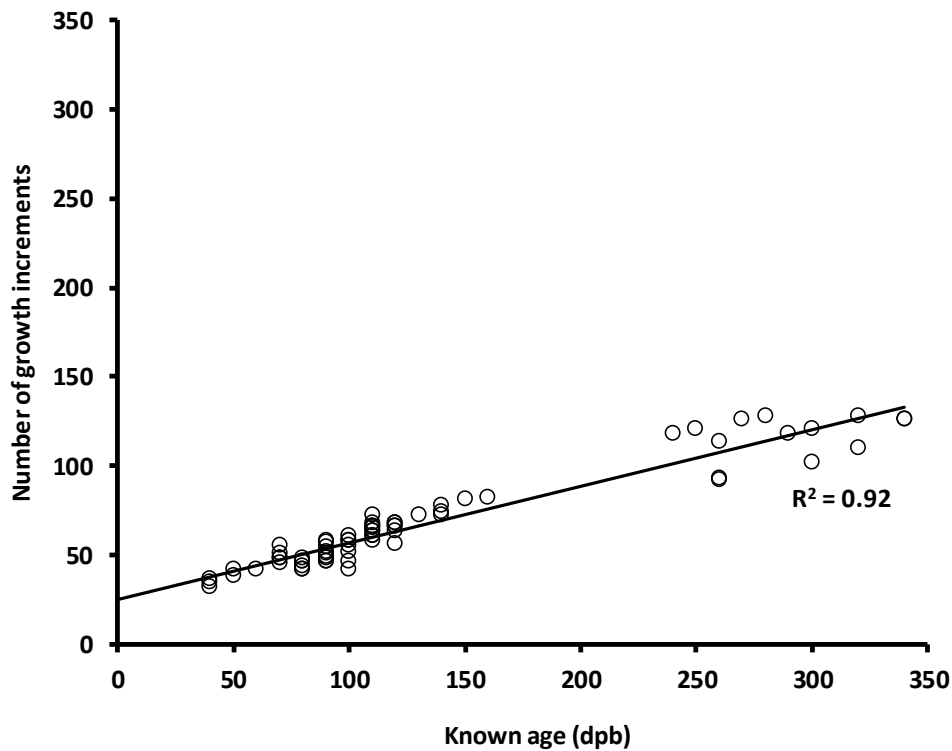


Fig. 3 Number of growth increments against known age with a fitted linear relationship.



There was a significant positive linear relationship between known age and increment counts (N=62,  $p<0.001$ ). This linear relationship was characterized by: known age =  $0.32 (\pm 0.01) \times$  number of growth increments +  $25 (\pm 1.76)$ . Known age data explained 92% of the variation in increment counts (Fig.3). This linear relationship was utilized to estimate the age of wild fish.

#### B) Growth modelling and mortality:

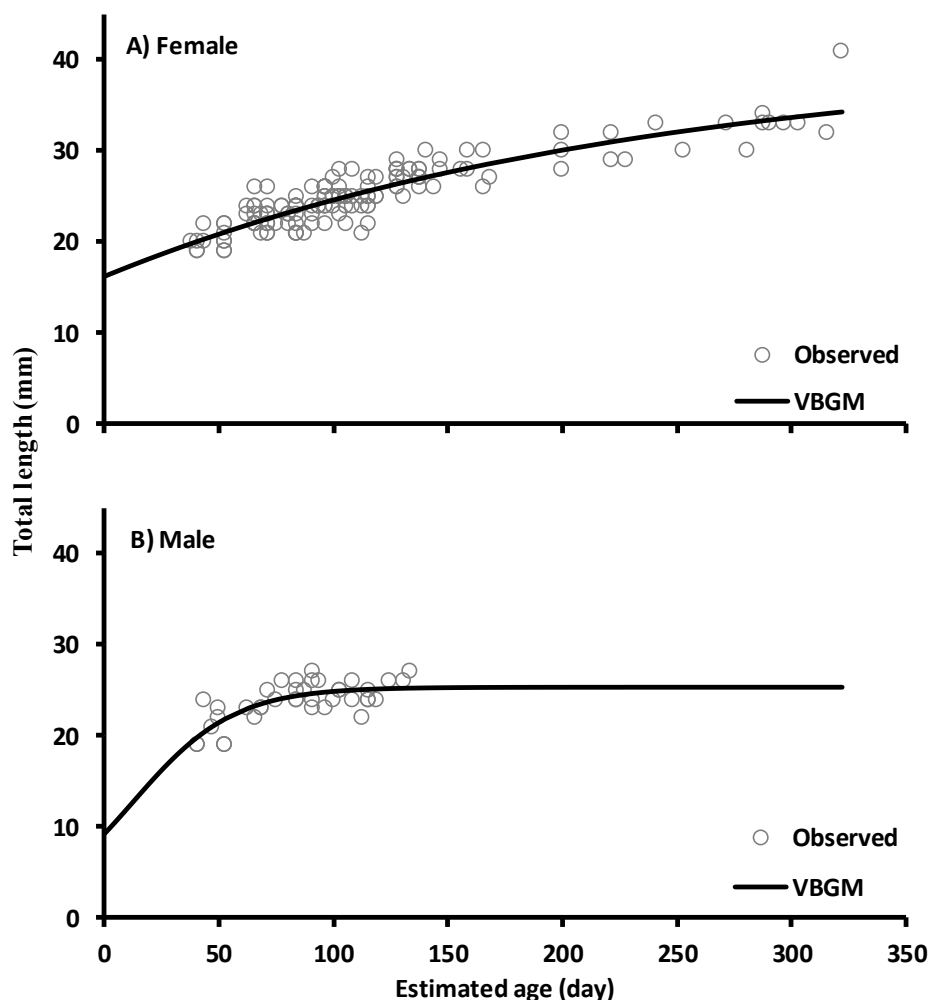


Fig. 4 Scatter plots of TL versus age data generated for wild fish with fitted VBGM.

Males reached an asymptote relatively quickly ( $\approx 75$  days), and they were relatively short-lived with no specimens exceeding 134 days of age (Fig.4). Additionally, females grew to significantly greater TL than males ( $t=16.5$ ,  $N=1432$ ,  $p<0.0001$ ).

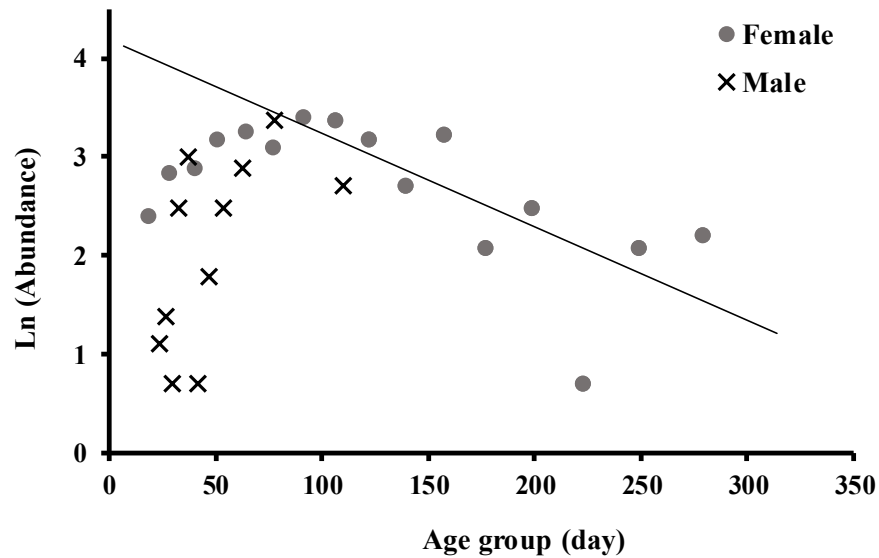


Fig. 5 Catch curve analysis generated for male and female *G. holbrooki* at the study site. Only the regression line for female data is shown.

Catch curve analysis revealed that the age-length key data of male fish was inadequate for estimating natural mortality due to a lack of older individuals; however, female data showed a descending trend in abundances of older groups (Fig.5).  $M$  was  $0.01 \text{ day}^{-1}$  for female *G. holbrooki*.

### C) Birthdates and growth rates:

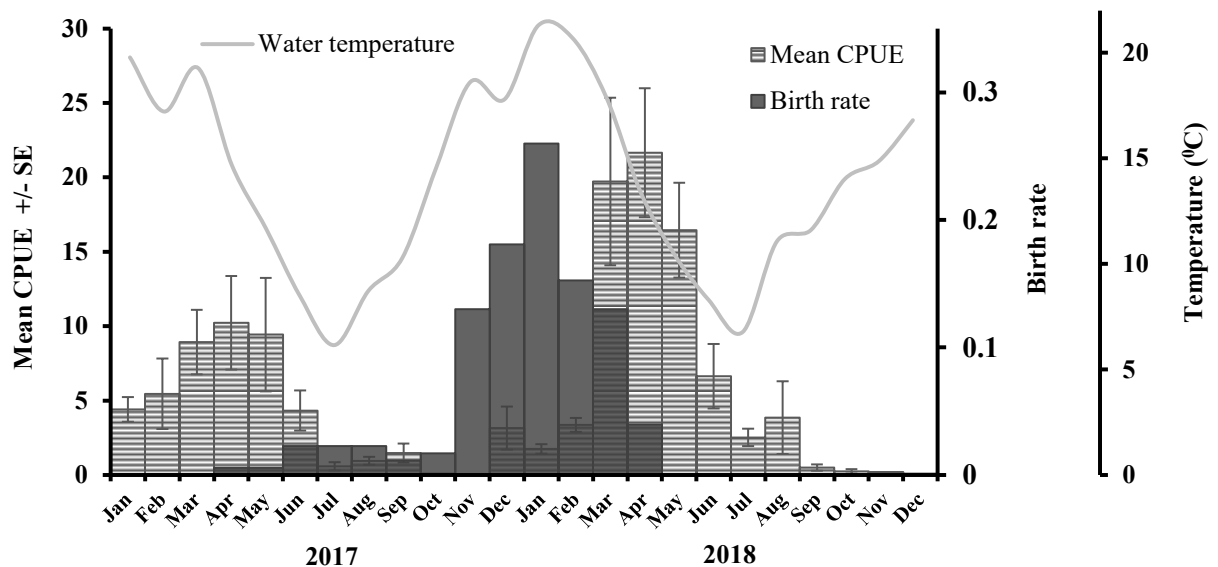


Fig. 6 Monthly frequency of birth (solid bars), average catch per unit effort (CPUE, lined bars with error bars  $\pm$  SE) generated for *G. holbrooki* and water temperature at the study site. Birth rate refers

to proportion of wild fish born in a given month, while mean CPUE corresponds to average number of fish captured per 25 m standard dip-net (mouth area of 0.1 m<sup>2</sup>) scoops for the month.

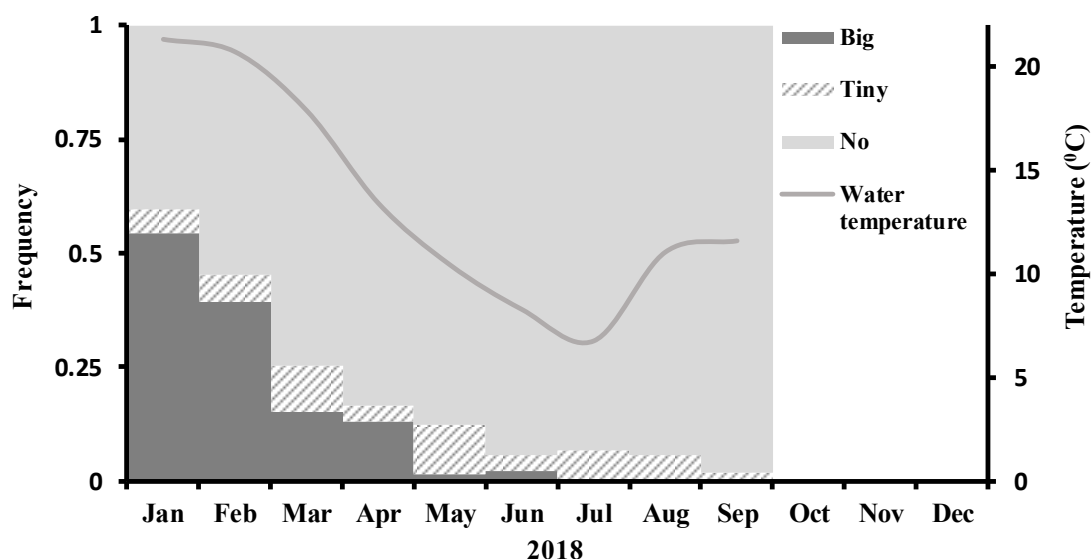


Fig. 7 Frequency of mature females with big, small, and no gravid spot in association with changes in water temperature.

The birth date of *G. holbrooki*, sampled between March to June 2018, ranged from April 2017 to April 2018. The birth rate was positively correlated with temperature ( $r=0.82$ ,  $p<0.001$ ) (Fig.6) as the frequency of newly birthed fish rapidly climbed from 0.016 in October 2017 to 0.129 in November 2017, then reached a peak in January 2018 (0.26) that coincided with an increase in temperature from 10.3°C (October) to 21.3°C (January). Following this, the birth rate gradually tailed off, corresponding with a decrease in temperature (Fig.6).

Although the trends of births and captures i.e., mean CPUE in 2018 were similar, there was a clear lag between the two, with peak births occurring in January while peak captures occurred three months later in April (Fig.6). A similar trend of peak captures in April of the preceding year (2017) was also evident, which presumably represents the time it takes the new cohorts to be fully selected by the gear used to capture them. Besides, the frequency of females with big gravid spots intensified in the summer months with a spike in January 2018 (Fig.7).

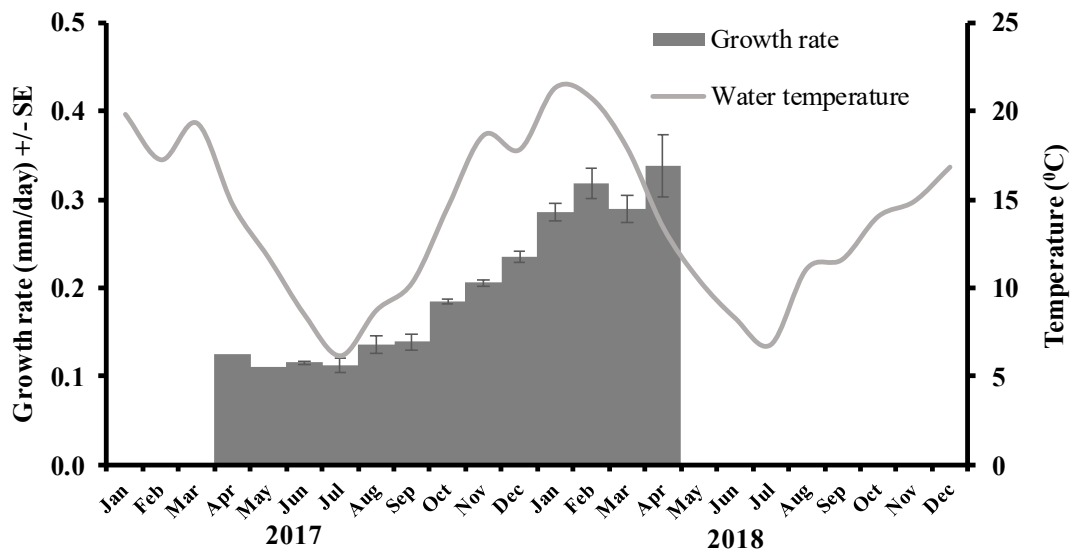


Fig. 8 Mean daily growth rates of *G. holbrooki* birthed in different months with corresponding changes in water temperature.

The mean daily growth rate significantly differed among cohorts birthed in different months ( $F=8.27$ ,  $N=193$ ,  $p<0.001$ ). The growth rate had a significant increase from  $0.14\pm0.009$  (mm/day) in September 2017 to  $0.19\pm0.002$  (mm/day) in October 2017, then fluctuated with an upward trend that crested at  $0.38\pm0.05$  mm/day in April 2018 (Fig.8).

Mean growth rates estimated for young cohorts (i.e., those birthed from October 2017 to April 2018) and over-winter cohorts (i.e., fish birthed from April to September 2017 which survived over winter) were 0.28 and 0.12 mm/day respectively.

## DISCUSSION

*Gambusia holbrooki* is considered a worldwide pest fish outside its native habitat. Most studies thus far have focused on its basic biology and the negative impacts it has on native species in the habitats it has invaded (Davies 2012; Lynch 2008; Pyke 2008). Despite its impact, there is a lack of fine-scale age structure data which limits the precise population-level insights necessary to develop intervention strategies to control this pest. The daily-age structure data, its validation, and its use to estimate key population parameters of the species in an invaded wetland, as presented here, is the first investigation of its kind for *G. holbrooki*. Our results will be particularly informative for assessing site-specific population parameters and vulnerabilities that can be exploited in control programs.

### Daily ageing improves ageing precision and growth modelling

In contrast to previous studies that used scales for ageing *G. holbrooki*, this study investigated otoliths as these are known to better preserve age records in bony fishes (Jones 1992). There are several disadvantages of using fish scales for age determination. Firstly, scales do not contain daily growth information (Jones 1992), a drawback that limits age inferences to years (e.g., 0+-year-old) rather than days. Secondly, scales are often prone to shedding and regeneration that can result in underestimations of age (Chilton and Beamish 1982; Jones 1992). This problem can be exacerbated in a highly combative species such as *G. holbrooki* which is behaviourally predisposed to scale-loss (Carmona Catot 2013). Moreover, resorption has been experimentally documented in calcified structures (including scales) other than otoliths (Jones 1992). Collectively, the use of otoliths results in greater precision in ageing compared to that of scales.

It has been previously reported that the majority (93.6–98%) of wild *G. holbrooki* are less than a year old (0+ year-old) (Cabral and Marques 1999; Carmona Catot 2013; Vargas and de Sostoa 1996), which is broadly in agreement with the findings of the present study (i.e., all fish were between 38–322 days old). Significantly, however, the use of otoliths instead of scales generated daily age data allowing fine-scale inferences and insights about the population dynamics of this notorious pest fish. The use of yearly age data, with limited age classes (i.e., 0+, 1+, 2+), often limits selection and comparative evaluation of growth models. For example, the use of year classes in the VBGM model not only resulted in coarse growth and population inferences in *G. holbrooki* (Erguden 2013; Scalici et al. 2007) but also precluded visual representation of data. In contrast, the present study, using daily age, facilitated fine-scale delineation of growth modelling for female and male *G. holbrooki*, which should allow better prediction of population dynamics of the species and management of their invasive populations, including monitoring efficacy of control options.

### **Mortality estimation**

The absence of older age classes in the male samples suggests that males may die out during the wintertime, which has also been hypothesized by earlier studies for this species in Tasmania (Lynch 2008). Despite the equal sex ratio at birth (Haynes and Cashner 1995), female-biased sex ratios observed in this study are in agreement with what has been commonly documented for the species in Tasmania (Keane and Neira 2004; Lynch 2008) and worldwide (Pyke 2005).

The lower number of males may negatively impact reproduction and natural recruitment at the population level. It is conceivable, nevertheless, that this influence may be negligible because *G. holbrooki* females can store sufficient sperm for up to eight broods (Constantz 1984), suggesting that females dictate the productivity of the population, i.e. a small number of females, surviving

over a harsh winter period, is capable of replenishing and sustaining high population densities through typical annual boom and bust population cycles. Also, it is possible that the males live as long as females despite their much smaller body size, and the observed sex ratio discrepancy and absence of older individuals may reflect capture bias associated with the relatively small size and agility of males, an aspect that cannot be completely ruled out and one that needs further investigation.

### **Birth rate and growth rate**

The present study appears to be the first work that has back-calculated the birthdate of *G. holbrooki* and used this information to pinpoint recruitment events. It was found that the frequency of newly birthed fish significantly differed between months with higher birth rates correlated with increased temperature. In particular, a significant increase in the number of births occurred when the mean temperature ranged from 14.5°C – 21.3°C, and births ceased when the temperature was less than 6.2°C at the study site. Correspondingly, previous studies also observed high recruitment of *G. holbrooki* within similar temperature ranges in Western Australia (15°C–16°C) (Pen and Potter 1991), Tasmania (14.6°C–19.5°C) (Keane and Neira 2004), and more generally during summer in many other parts of the world (Pyke 2005).

More importantly, the current study pinpointed a peak in reproduction during January 2018. This was corroborated by the frequency of gravid spots observed on females. Those with large gravid spots – likely to release young larvae (Norazmi-Lokman et al. 2016) – were most abundant in January 2018. The two findings collectively suggest that the most significant recruitment of *G. holbrooki* occurred in January. In contrast, the capture data indicated that the incursion peaked later in April 2018 at the study site, as was also supported by the preceding year's (2017) data. This discrepancy between the two peaks can be explained by the delay between birth and becoming susceptible to capture. The back-calculation of the birth date herein confirms that *G. holbrooki* populations collapse during the winter and rapidly increase when the weather becomes optimal, particularly in January. Such seasonal boom-and-bust cycles in *G. holbrooki* populations were also documented at the study site (Lynch 2008), suggesting a natural susceptibility which should be ideal for implementing targeted management measures, such as reproductive sabotage (Taylor et al. 2012).

Despite tolerance to a broad range of temperatures ( $\approx 6.2^{\circ}\text{C}$ – $35^{\circ}\text{C}$ ), cold temperatures result in the cessation of *G. holbrooki* growth and reproduction. Consistent with this, estimates of daily growth rate for cohorts birthed from October 2017 to April 2018 were typically higher than that of over-

winter cohorts (i.e., those individuals birthed in the previous season that survived over winter). The influence of age cannot be completely ruled out, however, minimal. Studies at the same location (Keane and Neira 2004), in Portugal (Cabral and Marques 1999), and in Spain (Vargas and de Sostoa 1996) have described similar patterns of variation in growth for this species.

### **Implications for pest control strategy**

Comprehensive knowledge of recruitment dynamics and scales of the incursion of pest species is fundamental to implementing effective strategies for their control. Although recruitment of *G. holbrooki* is known to occur during summer (Keane and Neira 2004; Macdonald and Tonkin 2008; Pyke 2005), there was a lack of predictive precision necessary for effective implementation of control measures.

This study, with its refined-technical approach, provides fine-scale insights about *G. holbrooki* recruitment cycles, precisely pinpointing that a peak reproduction event occurs in January at the study site. These insights have now provided valuable options for narrowing target periods for reproductive sabotage and physical removal at the study site, i.e., restricting access to reproductive habitats between December to February and targeting capture during winter months (July-August) when recruitment ceases, and populations levels are at the lowest. Likewise, an earlier study in Spain demonstrated a successful eradication of *G. holbrooki* concentrating on low-density conditions with the consolidation of intensive physical removal methods (Ruiz-Navarro et al. 2013). Similar spatiotemporal knowledge of recruitment dynamics has been successfully used to control pest populations of the common carp (Taylor et al. 2012).

Although the timing of boom-and-bust events may vary spatiotemporally within and between sites, a finer delineation of recruitment cycles suggests that the technique of examining otoliths can be employed to explore incursion scale and dynamics so as to control and possibly eradicate this pest fish elsewhere with greater efficiency whilst rationalizing often limited resources. Doing so will significantly reduce the complexity of the eradication program, lowering its cost, and thereby maximize the likelihood of success. This has led to focussed control efforts well in advance of January at the study sites, contrary to previous efforts from January to May when the numbers were

most abundant. Specifically, a relatively small targeted effort in removing over-wintering populations has already begun to delay summer recruitment, resulting in population decline.

Despite regulatory restrictions, there remain risks of passive new incursions of *G. holbrooki*, as was observed near the study site in 2016 (Inland Fisheries Service, personal communication), and also predicted by a habitat matching study (Davies 2012). Therefore, continued containment, control, and monitoring, employing integrated approaches are necessary to mitigate these risks here and elsewhere where *G. holbrooki* occurs. The daily-ageing process described in our study can play a critical role in determining site-specific recruitment dynamics allowing a tailored approach to the implementation of control and containment strategies that are simple and resource-effective. This ageing technique is also assisting in monitoring possible shifts in population dynamics and evaluating the impacts of the control measures. The approach and strategies can be easily applied to other invaded sites, leveraging similarly on the involvement of volunteers and citizen science programs.

In summary, the present study has for the first time demonstrated the practical use of otoliths in determining the daily age for *G. holbrooki*, a small, short-lived, and globally widespread invasive species. The techniques in this study facilitated fine-scale quantitative estimation of growth, mortality, and recruitment dynamics of this pest fish. Estimation of these biological processes, particularly birth rate, identified vital vulnerabilities that can be used for the control and management of this pest fish and could be readily applied to similar pest species (e.g., *G. affinis*) globally. Conceivably, the ageing technique is also readily adaptable and applicable to similarly small and short-lived fish species such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) that are popular models of vertebrate senescence and ageing.

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