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## Title Page

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## Abstract

Phenological responses to climate change have been widely observed and have profound and lasting effects on ecosystems and biodiversity. However, compared to terrestrial ecosystems, the long-term effects of climate change on species' phenology are poorly understood in aquatic ecosystems.

Understanding the long-term changes in fish reproductive phenology is essential for predicting population dynamics and for informing management strategies, but is currently hampered by the requirement for intensive field observations and larval identification. In this study, a very low-frequency sampling of juveniles and adults combined with otolith measurements (long axis length of the first annulus; LAFA) of an endemic Tibetan Plateau fish (*Gymnocypris selincuoensis*) was used to examine changes in reproductive phenology associated with climate changes from the 1970s to 2000s. Assigning individual fish to their appropriate calendar year class was assisted by dendrochronological methods (crossdating). The results demonstrated that LAFA was significantly and positively associated with temperature and growing season length. To separate the effects of temperature and the growing season length on LAFA growth, measurements of larval otoliths from different sites were conducted and revealed that daily increment additions were the main contributor (46.3%), while temperature contributed less (12.0%). Using constructed water-air temperature relationships and historical air temperature records, we found that the reproductive phenology of *G. selincuoensis* was strongly advanced in the spring during the 1970s and 1990s, while the increased growing season length in the 2000s was mainly due to a delayed onset of winter. The reproductive phenology of *G. selincuoensis* advanced 2.9 days per decade on average from the 1970s to 2000s, and may have effects on recruitment success and population dynamics of this species and other biota in the ecosystem via the food web. The methods used in this study are applicable for studying reproductive phenological changes across a wide range of species and ecosystems.

## Introduction

Alterations in reproductive phenology in response to climate change have been observed across a wide range of taxonomic groups, geographic regions and ecosystems (Walther *et al.*, 2002; Menzel *et al.*, 2006; Thackeray *et al.*, 2010; Gallinat *et al.*, 2015). Due to differences in phenological plasticity among individuals and species (Menzel *et al.*, 2006; Durant *et al.*, 2007; Vitasse *et al.*, 2009), asynchronous phenological changes have profound decoupling effects on both intra- and inter-specific interactions (Yang & Rudolf, 2010; Nakazawa, 2012; Sheriff *et al.*, 2013). These changes have the potential to affect population dynamics, community structure, ecosystem processes and the stability of ecosystems by changing recruitment success and resource match/mismatch relationships (Edwards & Richardson, 2004; Durant *et al.*, 2007; Miller-Rushing *et al.*, 2010). For example, sex differences in the phenological response to climate change can govern the dynamics of populations (Høye *et al.*, 2009; Sheriff *et al.*, 2013), and temporal mismatching between food resource availability and consumer reproduction can affect recruitment success of consumers and hence food web stability (Beaugrand *et al.*, 2003; Platt *et al.*, 2003).

In terrestrial systems, climate-driven changes in the arrival time of migrant birds, the appearance of butterflies and the flowering dates of garden plants have frequently been recorded because these taxa are easily identifiable and have received considerable attention from the public (Walther *et al.*, 2002). In aquatic systems, changes to thermal regimes (particularly changes in seasonality and thermal extremes) and intensification of the water cycle are important environmental consequences of global climate change. These changes can potentially result in an increase in the frequency and intensity of high and low precipitation and runoff events, and an amplification of warming through water vapour feedback (Huntington, 2006). However, the phenological responses of aquatic animals to these environmental changes is poorly understood (Ahas & Aasa, 2006; Thackeray *et al.*, 2010). Phenological changes of aquatic animals may occur rapidly over time (Burrows *et al.*, 2011) but are difficult to observe and monitor, requiring frequent and standardized field sampling to quantify

changes (Sims *et al.*, 2004; Greve *et al.*, 2005; Hovel *et al.*, 2017). Long-term surveys are rare because they are expensive and require the persistence from several generations of scientists (Winder & Schindler, 2004; Thackeray *et al.*, 2008). Fish reproductive phenology studies are often also hampered by larvae identification problems in the laboratory (Asch, 2015), since the larvae of many species overlap in their morphological characteristics (Ko *et al.*, 2013).

The fish otolith is an ideal recorder of fish life history events and influential changes to their environment (Pannella, 1971; Campana & Thorrold, 2001). By knowing the number of daily increments between the first annulus and the primordium (the initial complex structure of an otolith, reflecting the earliest stage of development), the relatively early or late birth among individuals of different year classes can be determined (Campana & Thorrold, 2001). This assumption requires that the periodicity of increments (daily and annually) are validated (Campana & Neilson, 1985), and assumes the environmental influence on annulus formation is relatively consistent among years (Pilling *et al.*, 2007; Millner *et al.*, 2011). Thus, by combining the environmental history and the otolith distance information, changes in fish reproductive phenology over time can be examined by a one-time sampling of juveniles and adults within different age classes based on the aforementioned assumptions. A key challenge of this method is how to accurately assign each fish into the correct calendar year class, particularly for long-lived species and old individuals. Recently, the application of dendrochronology (tree-ring) technologies (crossdating) to fish otoliths has successfully addressed this challenge (Morrongiello *et al.*, 2012; Black *et al.*, 2016), thus providing the opportunity to investigate changes in fish reproductive phenology from otoliths. Past fish otolith studies using dendrochronological technologies have focused on understanding the changes in growth width chronologies in response to environmental change (Morrongiello *et al.*, 2012; Black *et al.*, 2016; Izzo *et al.*, 2016; Ong *et al.*, 2016), but no one has used these methods to help quantifying changes in fish reproductive phenology.

The Tibetan Plateau (TP) is located in Central and East Asia, surrounded by the Earth's highest mountains including the Himalayas, Kunlun, Pamir, and Qilian. This vast region, with an area of approximately 2,500,000 km<sup>2</sup>, has been renowned as “the roof of the world” or “the third pole” for its high elevation (over 4,000 m a.s.l on average) and is recognised as an area sensitive to climate change (Kang *et al.*, 2010; Shrestha *et al.*, 2012). The TP has experienced an overall warming trend that is occurring at twice the rate of the average global trend during the last century (Liu & Chen, 2000; IPCC, 2014). Although terrestrial phenological changes in response to climate change on the TP have been reported (Yu *et al.*, 2010; Shen *et al.*, 2015), few studies have focused on aquatic ecosystem responses in this region. Increasing temperatures can stimulate earlier fish reproductive activities, as have been shown in a range of marine and freshwater systems (Ahas & Aasa, 2006; Asch, 2015). In addition, intensified water cycling due to increasing winter and spring precipitation (Kang *et al.*, 2010) has also changed riverine flow regimes on the TP (Cuo *et al.*, 2014). Changes in the timing of rising river flows and lake ice breakup, which is an important spawning cue for many freshwater fish species (Bunn & Arthington, 2002; Adrian *et al.*, 2009), may be expected to have important consequences for the reproductive phenology of freshwater fish on the TP. This reproductive phenological change should occur not only in riverine fish, but also in lacustrine fish, since fish from TP lakes (particularly saline lakes) must migrate to freshwater tributaries to complete reproduction (spawning and hatching) (Chen, 2000; Ding, 2012).

In this study, we used the long axis length of the first annulus (LAFA) on fish otolith sections as a proxy for the number of growing days for the young of the year (YOY) to examine long-term changes in the reproductive phenology of *Gymnocypris selinquoensis*, a long-lived, large-bodied freshwater fish species from a saline lake (Lake Siling Co) that is endemic to the TP. Using otolith dendrochronological technology, we assigned individuals into the correct calendar year class. We then linked LAFA changes of *G. selinquoensis* with climate change data from that period. We also decoupled the effects of daily increment additions (age) and temperature on otolith growth to examine the main source of variation in LAFA. We hypothesized that the reproductive phenology of

*G. selincuoensis* has advanced under climate change due to warming and flow regime changes, since this lacustrine species must migrate to tributaries to complete reproduction (Chen, 2000).

## Materials and Methods

### *Study area and species*

Lake Siling Co is the second largest lake on the TP (Figure 1) and has greatly expanded under climate warming over the past 40 years (39.4% by area, 1666.7 km<sup>2</sup> in 1976 to 2,323.6 km<sup>2</sup> in 2009; Meng *et al.*, 2012). It is a saline lake with salinity levels of approximately 20‰, and the maximum depth of the lake is over 40 meters (Chen, 2000). Lake Siling Co is the terminal lake in the largest closed drainage system (45,530 km<sup>2</sup>) on the TP (Guan *et al.*, 1984). The main drivers for its expansion are warming-induced glacial ablation coupled with increasing precipitation in the catchment area draining into the lake (Meng *et al.*, 2012). Four tributaries (freshwater) flow into this lake: Bochu Tsangpo (East), Tsachu Tsangpo (North), Tsagom Tsangpo (West, TT), and Ali Tsangpo (Southwest, AT) (Figure 1). The annual mean air temperature of this area was approximately 0 °C, and the mean annual total precipitation was approximately 300 mm over the past four decades. The annual mean air temperature and annual total precipitation have both markedly increased over the past forty years (Supplementary Figure S1).

*Gymnocypris selincuoensis* (Siling Co naked carp) belongs to the subfamily Schizothoracinae (Cyprinidae, Cypriniformes). It is the only carp species in Lake Siling Co and dominates the fish community. This species is characterized by degenerated scales, low growth rate, low fecundity and late sexual maturity as an adaptation to the high plateau environment, as in other Schizothoracinae species (Chen, 2000). The age of sexual maturity for males and females are seven and eight years old, respectively, and their life span can exceed thirty years (Chen, 2000; Chen *et al.*, 2002). The species is omnivorous, consuming a wide range of food sources, including detritus, algae, macrophytes, benthic invertebrates, phytoplankton, zooplankton and fish (Chen, 2000). The

reproductive season starts in late March or early April after winter ice thaws, and the main growing season is between April and October. Adults migrate to freshwater tributaries during reproductive seasons, while juveniles spend their first five years in rivers and grow in the lake thereafter (Chen, 2000).

### *Environmental variables*

Air temperature and precipitation data were obtained from the closest meteorological station (Pongok) of the Tibet Meteorological Administration and National Oceanic and Atmospheric Administration (NOAA, <http://gis.ncdc.noaa.gov/map/viewer/#app=cdo>). Water temperature is an essential trigger for fish reproduction; however, long-term monitoring of this variable was unavailable. To overcome this, water temperature was monitored on an hourly basis between November 2010 and May 2011 to establish a daily mean water-air temperature relationship. The water temperature probe (Apresys, APRESYS Inc., Duluth, USA) was fixed near the bottom of the water column (water depth was approximately 0.5 m), close to the mouth of the Bochu Tsangpo (31.7685°N, 89.3484°E) and the fish sampling sites. Only the water temperatures (> 0 °C) from April to May were used to establish a water-air temperature relationship, however, largely because the correlation was low in the winter. For example, air temperature in this area can fall down to -20 °C in the winter, whereas the water temperature will not fall below 0 °C due to its physical properties. In addition, *G. selincuoensis*, is likely to be more sensitive to environmental temperature changes during its reproductive period, since water temperature is a key environmental variable for fish gonad development and a cue for reproductive migration and spawning (Humphries & Walker, 2013). The relationship between the ten-day moving averages of both temperatures was also established, because only mean environmental temperatures maintained at a specific level for a week or more are likely to have biological significance (Campbell *et al.*, 1974). One or two days of occasional high temperatures are insufficient to trigger reproduction in the majority of the population. The low water temperature threshold for the commencement of *G. selincuoensis*



reproduction and growth is 2-3 °C (Ding, 2012). The changing trend of daily mean air temperature of the same Julian Day across decades was also investigated by averaging the daily data of all the years from each decade, and then smoothed by polynomial simulations.

#### *Field sampling, otolith preparation, crossdating and measurement*

In total, 544 *G. selincuoensis* juveniles and adults were captured from the mouth of the Bochu Tsangpo River (31.7676 - 31.7705°N, 89.3496 - 89.3473°E) by triple-layer gill nets and cast nets in 1997, 2010 and 2011. More details of these samples can be found in Tao *et al.* (2015). The sampling sites were close to each other and all located close to the river mouth where it flows into Lake Siling Co (Figure 1). Access constraints and safety considerations meant that sampling was only possible in this riverine area and not within the lake itself. Fish otoliths (lapilli) were removed from the utricle and cleaned and dried in the field. In the laboratory, otolith transverse sections were prepared using standard methods outlined in Tao *et al.* (2015). Briefly, the otolith was mounted on a glass slide using thermoplastic resin, ground transversely using sandpaper, and polished using films until they were close to the primordium. The section was then remounted with the polished surface down for bottom grinding and polishing. Sections were photographed under a microscope at 40× to 100× magnification depending on the section size. The periodicity of the annual increment and daily increment formation on lapilli has previously been validated (Chen *et al.*, 2002; Ding *et al.*, 2015). Therefore, the distance from the primordium to the edge of the first-year annulus was determined by the birth date and the length of the growing season of the corresponding calendar year.

We originally intended to use the major radius of the first annulus (MRFA, from the primordium to the edge tip of the first annulus, Figure 2b) as a growing-days indicator of YOY because its relatively long length was easy to measure and was also less affected by measurement error. However, due to the clarity of the primordium on some sections, we instead measured the long axis length of the first annulus (LAFA, Figure 2b). The LAFA measurements were conducted using ImageJ software (Schneider *et al.*, 2012), with assistance from the plugin 'IncMeas' (Rountrey, 2009). To assign each

Lafa to the correct calendar year, dendrochronological crossdating and 'list year' methods were used (Yamaguchi, 1991; Black *et al.*, 2016). Crossdating is a procedure that assumes environmental limitation would induce a synchronous pattern in increment width series of samples from a local population; thus, samples collected from different years can be cross-matched and each increment can be assigned to the correct year of formation (Black *et al.*, 2005). This procedure can ensure the precision and accuracy of dating, which are essential in temporal studies. Briefly, age was subtracted from the year of sampling to determine the birth year; therefore, each annulus could be assigned to the correct calendar year. Next, annual increment widths were measured along a line from the dorsal edge to the primordium. The increment width series were then detrended by a cubic spline for statistically confirming the crossdating results in COFECHA software (<http://web.utk.edu/~grissino/software>). For young individuals or old ones with insufficient series length measures to detrend in COFECHA, only the list year method was used to assign annuli to calendar years. COFECHA is a powerful tool to determine crossdating errors (Tao *et al.*, 2015).

Because of the relatively small sample size in early years (less than five fish otoliths for each year), we pooled Lafa samples into four decades after crossdating. One-way analysis of variance (ANOVA) was conducted to test the mean Lafa differences among decades, followed by Fisher's least significant difference (LSD) post-hoc test with significant level at  $P < 0.05$ . The mean Lafa of each year was also correlated with key environmental variables (i.e., air temperature and precipitation) using simple linear regression.

#### *Separating the contribution of increment number and temperature on otolith growth (otolith radius)*

The number of daily increments is largely determined by the length of the growing season, while the width of daily increments is highly associated with the environmental temperature (Ding, 2012). In natural systems, particularly those very sensitive to global warming (e.g. Siling Co), temperature could be the main factor affecting daily increment width of fish otolith, and potentially incorporated

with other factors such as food resources (Campana & Neilson, 1985; Schismenou *et al.*, 2016; Wenger *et al.*, 2016). However, it is almost impossible to retrieve data of historical food availability of *G. selincuoensis* each year, which relies on long-term and continuous monitoring on food resources. Therefore, this study assumes that LAFA of *G. selincuoensis* is mainly affected by growing season length and temperature. To determine whether growing season extension or temperature increase LAFA distance over time, we measured the otolith radius and counted the daily increments of *G. selincuoensis* larvae sampled from sites far apart. Larval fish sampling was conducted in May 2010 at three sites from Bochu Tsangpo (Bochu 1, Bochu 2 and Bochu 3), site 4 from Tsagom Tsangpo (Tsagom), and site 5 from Ali Tsangpo (Ali) (Figure 1, Supplementary Table S1). It was difficult to get access to all these sites at the end of growing season and to collect YOY due to the bad weather conditions and traffic. The larval otoliths were sectioned, then the radii were measured, and increments were counted with a light microscope at two magnifications (40x or 100x). More details about larvae otolith processing were described in Ding *et al.* (2015). One-way ANOVA and LSD post-hoc test ( $P < 0.05$ ) were conducted to detect any differences in the mean daily increment widths (MDIW) at different sites to evaluate the effect of variation in temperature on growth rates. Variation in larval otolith radius (LOR) as a function of both the number of daily increments (NoDI) and the mean daily increment width (MDIW) were estimated by multiple linear regression models. Therefore, the contribution of daily increment number on larval otolith radius ( $c$ ) could be estimated, and  $c$  equals to the explained variance (R-squared value) in LOR by NoDI.

#### *Estimating reproductive phenology changes*

Changes in reproductive phenology were estimated by correlating mean daily temperature to LAFA differences among decades. This approach relies on the assumption that the environmental temperature threshold for *G. selincuoensis* growth and reproduction was relatively stable over time, which is supported by previous studies (Chen, 2000; Pilling *et al.*, 2007; Millner *et al.*, 2011). Based on the information of the daily mean air temperature of the decades, the LAFA differences among

the decades, the mean MRFA/LAFA ratio ( $r$ ) and the contribution of the number of daily increments (age) on the larval otolith radius ( $c$ ), the growing season extension ( $GSE$ ) and the reproduction advancement ( $RA$ ) of *G. selincuoensis* were estimated. The calculation formula for  $GSE$  is:

$$GSE_{t1-t0} = (LAFA_{t1} - LAFA_{t0}) * r * c / MDIW$$

where  $t0$  is an early decade;  $t1$  is a late decade;  $LAFA_{t0}$  and  $LAFA_{t1}$  are the mean LAFAs of all samples of decade  $t0$  and  $t1$ , respectively. MDIW is the mean daily increment width of all larvae collected in May 2010.

Because the growing season extension ( $GSE$ ) could be due to both reproductive phenology advancement and delayed onset of winter ( $WD$ ),  $WD$  should be subtracted from  $GSE$  to estimate  $RA$ .  $WD$  was calculated from the Julian day (JD) at which the post-summer mean air temperature decreased to the threshold temperature for the two decades, while assuming that the environmental water temperature threshold for *G. selincuoensis* growth and otolith annuli formation was 2 °C (Ding, 2012). Daily mean air temperature of each year was firstly smoothed by polynomial simulations, then the JD (mean  $\pm$  SD) of each decade was estimated.

$$WD_{t1-t0} = JD_{t1} - JD_{t0}$$

The  $RA_{t1-t0}$  of the later decade ( $t1$ ) to the earlier decade ( $t0$ ) was then estimated by subtracting the days of winter delay ( $WD_{t1-t0}$ ) from  $GSE_{t1-t0}$ :

$$RA_{t1-t0} = GSE_{t1-t0} - WD_{t1-t0}$$

## Results

### *Relationship between water and air temperatures and warming trends*

Mean water temperature data and air temperature data over 65 days (from 1 March to 4 May 2011) were used to establish the relationship between these two variables (Supplementary Figure S2a). The daily mean water temperature was significantly associated ( $P < 0.01$ ,  $R^2 = 0.35$ ) with the daily

mean air temperature record from the closest meteorological station (Pongok; Supplementary Figure S2a). The relationship between the ten-day moving averages of both temperatures was also significant ( $P < 0.01$ ,  $R^2 = 0.37$ , intercept = 1.16, slope = 0.73; Supplementary Figure S2b). The water temperature was slightly higher than the air temperature during this period, such that when the ten-day mean air temperature was 1 °C, the ten-day mean water temperature was approximately 2 °C. Therefore, the assumed environmental air temperature threshold for *G. selincuoensis* growth and otolith annuli formation was 1 °C.

The analysis of decadal changes in daily mean air temperature revealed that air temperature gradually increased from the 1970s to the 2000s (Figure 3). In addition, both the highest mean daily air temperature in summer and the lowest mean daily air temperature in winter increased by 1.5 °C and 4.4 °C, respectively. Decadal changes were also clearly evident in the timing and overall duration of days with elevated temperature, occurring progressively earlier and for longer periods over time. For example, the Julian date at which the post-winter mean air temperature increased to 1 °C arrived progressively earlier over the decades (day 119.5 in the 1970s versus day 114.5 in the 2000s, Supplementary Table S2). The date at which post-summer mean air temperature decreased to 1 °C was very similar for the first three decades but occurred 5 days later in the 2000s compared to the 1970s (Supplementary Table S2). The delayed onset of winter (*WD*) among decades was calculated based on these data (Supplementary Table S2).

#### *LAFAs changes over time and the relationship with environmental variables*

Among the 544 otolith sections of juveniles and adults examined, 153 of them were excluded from measurement due to poor resolution of annual increments. Of the 391 qualified samples, 111 samples were accurately crossdated with the assistance of COFECHA. Two crossdating errors were

identified and corrected by COFECHA. The birth years of the remaining 280 samples were determined from the 'list year' method. The mean LAFA was  $719 \pm 118 \mu\text{m}$  (mean  $\pm$  SD) and significantly increased from the 1970s to 2000s (Figure 4). The LAFA series also showed a gradual and significant increase from the 1970s to 2000s (Supplementary Figure S3).

Simple linear regression between the mean LAFA of each year and environmental variables found that LAFA was positively and significantly associated with annual mean air temperature, growing season mean air temperature, and days of mean air temperature over  $1^\circ\text{C}$  ( $P < 0.001$ , Figure 5a, b, and c). The annual mean air temperature explained the most (58%) variation in LAFA (Figure 5a). In comparison, total annual precipitation was not significantly correlated with LAFA and thus could not explain any of the LAFA variability (Figure 5d).

#### *Contributions of the number of increments and temperature to otolith growth*

The mean daily increment width (MDIW) of larval otolith samples was significantly different among sites ( $F = 46.63$ ,  $P < 0.001$ ; Table 1). In general, the MDIWs of samples from Bochu Tsangpo were significantly higher than those of samples from Ali Tsangpo and Tsagom Tsangpo ( $P < 0.05$ ). All the linear regression models of larval otolith radius (LOR) with the number of daily increments (NoDI) and with the mean daily increment width (MDIW, an indicator of environmental temperature) were significant (Table 2). The NoDI and MDIW collectively explained 71.1% of the variance in LOR of *G. selincuoensis* larvae (model 1), while the NoDI and the MDIW alone explained 46.3% (c) and 12.0% of the variance in LOR, respectively.

#### *Changes in reproductive phenology*

The observed LAFA increase due to daily increment additions (or growing season extension, GSE) was 16.5 days from the 1970s to 2000s (Figure 6). To match the estimated daily increment addition with the mean environmental temperature (Figure 3) or delay onset of winter (WD, Supplementary Table S2), the reproduction date of *G. selincuoensis* would have occurred an average of 2.9 days

(11.7 days divided by four) earlier per decade on average from the 1970s to 2000s, and the growing season of *G. selincuoensis* was extended 3.0 days towards winter from the 1990s to 2000s but not as much (1.8 days) during the 1970s and 1990s (Figure 6, Supplementary Table S3).

## Discussion

### *Evidence for advanced reproduction in G. selincuoensis*

This study has shown that the growth (as indicated by LAFA) of *G. selincuoensis* YOY increased from the 1970s to 2000s and was significantly and positively correlated with annual mean air temperature and length of growing season (indicated by days over 1 °C). Variation in the LAFA can be mainly attributed to the addition of daily increments (46.3%) and secondarily to increasing environmental temperatures (12.0%), based on larval otolith measurements (Table 2). This implies that the increased LAFA was mainly due to the extension of the growing season. Assuming that the environmental temperature threshold for *G. selincuoensis* growth and otolith annuli formation was relatively stable through time (Chen, 2000; Pilling *et al.*, 2007; Millner *et al.*, 2011; Ding, 2012), we can conclude that the reproductive phenology of *G. selincuoensis* advanced between the 1970s and 2000s, thus supporting our hypotheses as expected.

To the best of our knowledge, this is the first phenological study of potential climate change responses in aquatic ecosystems on the Tibetan Plateau. The reproductive phenology of *G. selincuoensis* was significantly advanced during the period of the 1970s to the 2000s and supports other studies that document advancing reproductive activities of fish under global climate change. For example, the positive correlation between the date of the YOY cohort first captured and the date of ice breakup indicated an earlier spawning of three-spine stickleback (*Gasterosteus aculeatus*) from a boreal lake experiencing climate change (Hovel *et al.*, 2017). In the North Atlantic, the initiation of smolt (*Salmo salar*) seaward migration occurred 2.5 days earlier per decade from 1961

to 2010 (Otero *et al.*, 2014). During the period from 1948 to 1999, the spring phenological phases of fishes in Estonia have advanced from 10 to 30 days under global warming (Ahas & Aasa, 2006).

The advancement of *G. selincuoensis* reproduction mainly occurred from the 1980s to 1990s, which corresponded to the accelerated warming of the TP (Shen *et al.*, 2015). However, compared to the 1990s, the extended growing season of *G. selincuoensis* was largely the result of the delayed onset of winter and not reproductive advancement in the 2000s (Figure 6). Other studies on the TP also found similar advanced spring phenology in terrestrial ecosystems across the TP during the 1980s and 1990s (Shen *et al.*, 2015), but spring phenologies were not advanced or were even delayed in the 2000s (Yu *et al.*, 2010; Shen *et al.*, 2013). For example, the vegetation green-up significantly advanced by 8.8 days per decade from 1982 to 1999 but was marginally delayed from 1999 to 2006 (Piao *et al.*, 2011). Hence, the results of our study are consistent with previous studies mentioned above and shows that our analyses were successfully able to partition the effects of reproductive advancement versus the delayed onset of winter, which seemed to occur in different decades.

#### *Potential reasons for, and consequences of, G. selincuoensis reproductive phenology advancement*

Our study indicated that earlier warming of lake water could be the determinant of *G. selincuoensis* reproductive phenology advancement. This is consistent with findings from other analogous studies around the world. For example, the timing of the appearance of summer-spawning larval fish in the Western English Channel was significantly dependent on the sea temperature before spawning, with earlier spawning during warmer years (Genner *et al.*, 2010). Similarly, the earlier timing of the occurrence of fish larvae in the North Sea was significantly correlated with warmer sea surface temperatures during the preceding winter (Greve *et al.*, 2005).

There are several potential explanations for this phenomenon. First, increases in the temperature can accelerate the metabolic rate of organisms and in turn, accelerate growth and gonad



development, thus inducing advancement of reproductive migration and spawning. For instance, the gonad maturation of both male and female Pacific Herring (*Clupea harengus pallasii*) was related to daily sea temperature, and warmer conditions resulted in earlier spawning (Ware & Tanasichuk, 1989). Second, water temperature is an important cue for synchronizing fish reproduction with the seasonal increase in abundance of food (Sims *et al.*, 2004). Increases in the environmental temperature earlier in the year can cause advancement in the stimulation of primary and secondary production, for example, the blooming of phytoplankton and zooplankton (Mackas *et al.*, 1998; Weyhenmeyer *et al.*, 1999; Edwards & Richardson, 2004). Early warming can not only result in the early availability of autochthonous food sources but also allochthonous organic matter (e.g., terrestrial plants and insects) inputs into rivers and lakes. Chen *et al.* (2013) found that climate warming on the TP substantially increases terrestrial primary production. These allochthonous sources can be transported into water through land surface runoff and provide a food subsidy for aquatic organisms such as the omnivorous fish *G. selincoensis*. Temperature-induced early spawning and new recruits would be able to exploit the earlier availability of food. Third, climate warming also results in earlier spring flow (Cuo *et al.*, 2014). The timing of rising flows and temperatures is another factor that cues reproductive migration and spawning in many fish (Bunn & Arthington, 2002). In this area, earlier spring flows could result from changing precipitation regimes and early glacial melting (Cuo *et al.*, 2014; Kuang & Jiao, 2016).

Reproductive phenology advancement not only has effects on the demographic dynamics of *G. selincoensis* (Miller-Rushing *et al.*, 2010) but could also have further effects on other members of the food web (Moore & Schindler, 2010). Climate change is likely to have extended the growing season of *G. selincoensis* YOY in both directions through time; i.e., advancing reproduction in spring and delaying onset of winter. Larvae of *G. selincoensis* that experience a longer growing season can reach to a larger size before the first winter, thus substantially reducing the overwinter mortality of YOY (Teal *et al.*, 2008; Ding, 2012). Therefore, advanced reproduction could result in *G. selincoensis* population recruitment success and increases in population size. This represents an increase in food

availability for piscivorous birds (i.e., *Larus brunnicephalus*, *Tachybaptus ruficollis*, and *Phalacrocorax carbo*). In Qinghai Lake, the availability of fish resources might be the determinant of reproductive success for *P. carbo* (Zhang *et al.*, 2007). On the other hand, we know little about the phenological changes in the food sources (phytoplankton, zooplankton, and benthic algae and invertebrates) of *G. selincuoensis*. If changes in the food source availability are not coupled with reproductive phenology changes in *G. selincuoensis* under climate change (Wiltshire *et al.*, 2008), the mismatch between food availability and *G. selincuoensis* reproduction could cause higher larval mortality (Durant *et al.*, 2007; Post & Forchhammer, 2008). For example, in North Sea, the recruitment success of lesser sandeel (*Ammodytes marinus*) was found to depend on the degree of synchrony between sandeel larval hatching and egg production of their copepod prey (Régnier *et al.*, 2017).

#### *Dendrochronological methods to study phenological changes and other effects of climate change*

The methods applied in this study enabled us to make use of a low-frequency sampling on fish juveniles and adults to investigate reproductive phenological changes, which is an effective way to compensate long-term continuous field observation and overcomes larval identification (Sims *et al.*, 2004; Ko *et al.*, 2013; Asch, 2015). One limitation of this study is that partitioning larval otolith growth due to temperature increase versus growing season length were not based on the entire growing season of YOY (as larvae could not be collected from all sites at the end of the growing season). This may introduce error into the estimation of growing season extension and reproductive advancement and requires more research to understand the relative contribution of temperature and growing season length on otolith growth. Another potential limitation in this study is that we assumed that temperature was the key factor influencing otolith growth and we did not consider other factors such as food, which may be important in some circumstances (Hinrichsen *et al.*, 2010; Schismenou *et al.*, 2014). In spite of these limitations, the methods provide opportunities for further applications in exploring reproductive phenology changes in fish and other taxa (e.g., bivalves,

gastropods) in a range of ecosystems. In addition, a very recent terrestrial study on the TP has also proposed comparable methods of using tree ring microstructure chronology data to examine changes in spring vegetation phenology (Yang *et al.*, 2017). Therefore, dendrochronological technologies have promising application potentials in studying both aquatic and terrestrial phenology, in addition to other effects of climate change.

Adaptation, migration and death are three main alternatives for organisms facing climate changes and are expressed as changes in growth and phenology, distribution, and mortality, respectively. All these biological responses to climate change are difficult to examine but potentially become achievable with the application of dendrochronological methods. For example, dendrochronological technologies (crossdating) have been widely applied to establish accurate long-term growth chronologies and to explore the effects of climate change on the growth of aquatic animals, such as fish, bivalves, and corals (Morrongiello *et al.*, 2012; Black *et al.*, 2016). With the assistance of dendrochronological methods and historic environmental records, this study successfully investigated long-term changes in *G. selincuoensis* reproductive phenology using low-frequency sampling of juveniles and adults. Biochronologies of stable isotope data or trace element data in fish otoliths, bivalve shells, and other hard parts, combined with “isoscape” information or trace element backgrounds of the environment (Campana, 1999; Bowen *et al.*, 2009; Sturrock *et al.*, 2012), can potentially reveal the movement history of animals, and then allow the identification of changes in their distribution range under climate change. Moreover, dendrochronological methods can contribute to identifying massive mortality from extreme climate events. For example, mass mortality events of geoducks (*Panopea generosa*) on the Vancouver Island were identified by comparing updated and dead geoduck chronologies, and associating the results with storm-induced burial (Lohead *et al.*, 2012). Therefore, dendrochronological methods, including crossdating, could be powerful and effective tools for exploring the effects of climate change, which is difficult to achieve through long-term traditional sampling and monitoring.

In conclusion, this study showed that the LAFA of *G. selincuoensis* YOY significantly increased from the 1970s to 2000s under climate change, largely due to a growing season extension and secondarily to temperature increases. The results strongly indicate that the reproductive phenology of *G. selincuoensis* has advanced during this period. The change in reproductive phenology of *G. selincuoensis* that was detected is also significant in predicting responses of the other biota in Lake Siling Co and other ecosystems on the TP. The methods applied in this study can potentially be used in phenological studies for other taxa. Furthermore, dendrochronological methods can facilitate the understanding of the effects of climate change at all the three key response aspects, including adaptation, migration, and mortality.

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## Tables

Table 1. Mean daily increment width ( $\pm$  SD) of larval otoliths from different sites. *P* values for LSD post-hoc test between site pairs are also shown.

Site	N	Daily Increment Width (Mean $\pm$ SD)	Bochu 1	Bochu 2	Bochu 3	Tsagom	Ali
Bochu 1	91	3.56 $\pm$ 0.42	1				
Bochu 2	49	3.59 $\pm$ 0.28	1	1			
Bochu 3	70	3.70 $\pm$ 0.34	0.124	1	1		
Tsagom	19	2.60 $\pm$ 0.28	< 0.01	< 0.01	< 0.01	1	
Ali	18	3.00 $\pm$ 0.27	< 0.01	< 0.01	< 0.01	< 0.01	1
Total	247	3.49 $\pm$ 0.47					

Table 2. Multiple linear regression models of larval otolith radius (LOR) with the number of daily increments (NoDI) and with the mean daily increment width (MDIW).

	N	R <sup>2</sup>	F	Sig	Dependent Variable	Predictor	Unstandardized Coefficient	Sig
Model 1	247	0.711	299.705	< 0.01	LOR	Constant	-101.534	< 0.01
						NoDI	2.886	< 0.01
						MDIW	34.347	< 0.01
Model 2	247	0.463	211.371	< 0.01	LOR	Constant	30.514	< 0.01
						NoDI	2.502	< 0.01
Model 3	247	0.120	33.551	< 0.01	LOR	Constant	28.050	0.049
						MDIW	23.447	< 0.01

### Figure captions

Figure 1. Study area and sampling sites for this study. The black star shows the sampling site for the *Gymnocypris selincuoensis* reproductive phenology study. Black dots show the five sites where *G. selincuoensis* larvae were collected for assessing the contribution of daily increment additions and environmental temperature on otolith growth. Sites 1, 2, and 3 are from the Bochu Tsangpo River; site 4 is from Tsagom Tsangpo (TT); and site 5 is from Ali Tsangpo (AT). The latitude and longitude data for each site are included in Supplementary Table S1.

Figure 2. Sectioned otolith of *Gymnocypris selincuoensis* showing (a) annuli (white dots), (b) the major radius of the first annulus (MRFA) and the long axis of the first annulus (LAFA) and primordium (P, white arrow).

Figure 3. The changing trend of daily mean air temperature on a decade scale from the 1970s to 2000s. The thin lines show the Julian day mean air temperature for each decade. The smoothed lines show polynomial simulations of the decadal averages. The dashed line is the 1 °C threshold line for *G. selincuoensis* growth and otolith annuli formation.

Figure 4. Box plot of decadal changes in the long axis length of the first annulus (LAFA) on *Gymnocypris selincuoensis* otolith sections. Boxes with different small letters indicate significant differences at  $P < 0.05$  (One-way ANOVA,  $F = 36.39$ ,  $P < 0.001$ ). The numbers in the brackets indicate the sample size for each decade. Each dot indicates a sample, and data from each decade were fitted to a normal distribution (indicated with vertical curved lines).

Figure 5. Linear regression between the long axis length of the first annulus (LAFA) on *Gymnocypris selincuoensis* otolith sections and environment variables: (a) annual mean air temperature, (b) growing season mean air temperature, (c) days with mean air temperature over 1 °C for each year, and (d) annual precipitation. Samples from each decade are indicated with coloured symbols.

Figure 6. The relative days (mean  $\pm$  1SE) of the growing season extension, delay in onset of winter and reproduction advancement in spring for *Gymnocypris selincuoensis* young of the year (YOY) among decades. Data calculations were based on decadal mean values of LAFA and decadal mean air temperatures, with the 1970s set as the reference decade.









