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Sensitive males: inbreeding depression in an endangered bird

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Attempts to conserve threatened species by establishing new populations via reintroduction are controversial. Theory predicts that genetic bottlenecks result in increased mating between relatives and inbreeding depression. However, few studies of wild sourced reintroductions have carefully examined these genetic consequences. Our study assesses inbreeding and inbreeding depression in a free-living reintroduced population of an endangered New Zealand bird, the hihi (Notiomystis cincta). Using molecular sexing and marker-based inbreeding coefficients estimated from 19 autosomal microsatellite loci, we show that (i) inbreeding depresses offspring survival, (ii) male embryos are more inbred on average than female embryos, (iii) the effect of inbreeding depression is male-biased and (iv) this population has a substantial genetic load. Male susceptibility to inbreeding during embryo and nestling development may be due to size dimorphism, resulting in faster growth rates and more stressful development for male embryos and nestlings compared with females. This work highlights the effects of inbreeding at early life-history stages and the repercussions for the long-term population viability of threatened species.

\textbf{Keywords:} hihi; inbreeding; lethal equivalents; reintroduction; offspring survival

\section{1. INTRODUCTION}

The concept of inbreeding depression has played a central role in evolutionary thinking for more than a century (Darwin 1876; Wright 1922) and has increasingly become a concern to conservation biologists (Frankel 1974). Threatened species are often characterized by their small and isolated populations, which may also be declining, such as populations of island-dwelling birds (Caughley & Gunn 1996). These attributes make them more vulnerable to inbreeding owing to the higher chances of mating with relatives and the effects of genetic drift (Keller & Waller 2002). Reintroduced populations are particularly susceptible to inbreeding because reintroduction involves establishing populations with small numbers of founders, often of already rare species. Reintroductions are increasing in frequency, yet they remain controversial, partly because genetic analyses of the source and reintroduced populations are required for effective management strategies but are rarely undertaken (Armstrong & Seddon 2008; Jamieson 2009).

The loss of fitness owing to inbreeding (known as inbreeding depression) is caused by the expression of recessive deleterious alleles and increased homozygosity (Keller & Waller 2002). It has been found extensively in domestic and laboratory animals, and in many captive populations of wild animals (Lynch & Walsh 1998). In wild populations, empirical evidence of inbreeding depression has proved more elusive, partly owing to the natural dispersal mechanisms that have evolved to minimize mating between relatives (Pusey & Wolf 1996), but also because of the difficulties of acquiring the long-term breeding and life-history data on marked individuals needed to calculate inbreeding and test for inbreeding depression (Pemberton 2008). In addition, the low frequency at which close inbreeding generally occurs leads to small sample sizes that reduce the power of statistical analysis (Kruuk et al. 2002). However, studies of wild populations are increasing and have shown that inbreeding depression occurs widely and can be exacerbated under natural conditions (Crnokrak & Roff 1999; Keller & Waller 2002).

Island bird species are particularly suitable for detailed genetic and ecological studies as they can be readily marked and their survival and reproduction monitored. Inbreeding is known to depress survival in a number of island populations of wild passerines (Van Noordwijk & Scharloo 1981; Keller 1998; Keller et al. 2002; Kruuk et al. 2002; Jamieson 2009). The magnitude of the effect is dependent on the stage of development measured (embryo, nestling, fledgling and adult), the sex of the individual (Smith et al. 2006), the parental level of inbreeding (Van Noordwijk & Scharloo 1981; Keller 1998), and the demographic and environmental conditions to which the individual is exposed (Crnokrak & Roff 1999). In addition, it has been argued that island species experience population bottlenecks that may purge the genetic load, therefore attenuating the effects of inbreeding (Hedrick & Kalinowski 2000). However, little support has been found for the purging of deleterious alleles after bottleneck events (Briskie & Mackintosh 2004). Furthermore, lowered hatching success has been linked to embryo mortality owing to

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inbreeding (Greenwood et al. 1978; Bensch et al. 1994) as major genes are expressed at early developmental stages (Keller & Waller 2002). Nestlings and fledglings may also harbour a considerable inbreeding load that affects their survival probability (Greenwood et al. 1978; Van Noordwijk & Scharlau 1981; Keller 1998; Kruuk et al. 2002), and this fitness effect may have serious consequences for overall population viability (Crnokrak & Roff 1999; Hedrick & Kalinowski 2000; Keller & Waller 2002).

One of the major difficulties encountered in investigating inbreeding depression in natural populations is obtaining accurate estimates of individual inbreeding coefficients. Traditionally, inbreeding is calculated from multigenerational, complete (no missing individuals) and accurate (no misassigned parentage) pedigrees (Pemberton 2008), which are not available for most natural populations. In the absence of a reliable pedigree, an alternative approach is to infer inbreeding coefficients from individual variation in molecular markers. Marker-based methods have been used to estimate average heterozygosity as a proxy for inbreeding (Ballonx et al. 2004), relatedness between individuals (Bensch et al. 1994) and point estimates of inbreeding (Glémion et al. 2006) to address inbreeding depression (Keller & Waller 2002). Using the same population genetics principles (i.e. probability of identity by descent) to estimate relatedness, the inbreeding coefficient of an individual can be estimated from its genotype and allele frequencies at a set of marker loci (e.g. Ritland 1996; Wang 2007).

In this study, inbreeding coefficients estimated from marker data are used to investigate inbreeding depression in the reintroduced population of New Zealand hihi (or stitchbird, Notiomystis cincta) on Tiritiri Matangi Island (220 ha). The Tiritiri Matangi population of hihi was established in 1995 via translocation of individuals from the remnant source population on Little Barrier Island (3020 ha; Ewen & Armstrong 2007). The Little Barrier population numbers somewhere in the hundreds to low thousands of individuals (Taylor et al. 2005) but is thought to have undergone more substantial population bottlenecks owing to predation and disease (Angehr 1984). The founding of Tiritiri Matangi’s hihi population also constitutes a bottleneck. The small population size following establishment of hihi can increase the rate at which inbreeding accrues, and this can lead to inbreeding depression (Frankham et al. 2002).

Here, we test for the effect of inbreeding at two key stages of development from embryo to nestlings in a single breeding season of hihi. We predict that inbreeding effects will be observed more strongly on embryos and nestlings as this is a period of rapid and energetically expensive development. We also test for an interaction between inbreeding depression and offspring sex given size dimorphism in offspring hihi (Ewen et al. 2009). To enable comparison with other studies, we systematically measure the level of inbreeding depression by estimating the number of lethal equivalents (Morton et al. 1956).

2. MATERIAL AND METHODS

(a) Study species and population

The hihi is an endangered forest-dwelling passerine. Hihi were originally found throughout the northern half of New Zealand, but following European colonization they declined to a single remnant population on Little Barrier Island, around the year 1885. Its near-extinction has been attributed to the combined effects of exotic predators, disease and habitat loss (Armstrong et al. 2002). Conservation of hihi focuses on the establishment of new populations via reintroduction (Armstrong et al. 2002). Fifty-one hihi were reintroduced to Tiritiri Matangi Island (36°36’S, 174°53’E) in 1995 and 1996, although the known survivors of reintroduction numbered less than 20 (Ewen et al. 1999). The hihi population on Tiritiri Matangi is currently estimated to grow by about 34 per cent per annum in the absence of harvesting (Armstrong & Ewen submitted). However, harvesting of individuals to supply new reintroductions has stalled population growth, and population size now sits at a managed carrying capacity of approximately 150 adults.

During the breeding season from September to February, hihi are socially monogamous and territorial, but highly promiscuous. Hihi breed annually and have overlapping generations. Their mean life expectancy is 4 years, but some individuals have been known to survive to 9 years of age. Extra-pair copulations are frequent, obvious and often forced on females by males (Castro et al. 1996), and result in substantial extra-pair paternity (Ewen et al. 1999; Castro et al. 2004). Male hihi are larger and more colourful than females (males approx. 40 g; females approx. 32 g), with size dimorphism appearing early in development with sex-specific nesting growth trajectories (Ewen et al. 2009).

(b) Sampling

Nests were monitored daily from building through to fledging of chicks. Hihi are cavity nesters and on Tiritiri Matangi almost exclusively nest in artificial boxes. Females lay between one and four clutches per season (mean clutch size = 4 eggs), but can rear a maximum of only two broods annually (Taylor et al. 2005). The incubation period spans 14 days and fledging occurs approximately 30 days after hatching. Most individuals in the population were measured and weighed as nestlings at 21 days of age, when they were also given a uniquely numbered metal band and combination of colour bands. In addition, a small blood sample was collected via brachial venipuncture and stored in 95 per cent ethanol. All unhatched eggs were collected 3 days post-expected hatching date and dead nestlings were recovered during nest checks. Unhatched embryos and tissue samples from dead nestlings were preserved in 95 per cent ethanol. Finally, any birds not caught in the nest were captured and blood-sampled via brachial venipuncture as adults.

(c) Genetic analysis and inbreeding estimates

Genomic DNA was extracted from tissue and whole blood using the ammonium acetate precipitation method (Nicholls et al. 2000). All individuals were genotyped at a set of 19 autosomal selectively neutral microsatellite loci, which are widely distributed in the passerine genome (see Brekke 2009). Samples were individually amplified twice and if the allele calls were not consistent they were repeated until they were (estimate of genotyping errors across all markers: 0.002). The forward primer of each set was fluorescently labelled and products were analysed on an ABI sequencer. Genetic diversity in this population is known to be high (allelic richness: 5.11; observed heterozygosity: 0.66; Brekke 2009). All individuals were sexed using two different sex-typing loci (n = 221; Z-002A, Dawson 2007; Z-037B, D. A. Dawson 2002, unpublished data) or using
morphology based on plumage coloration ($n = 13$, including four individuals with no available genotypes).

The triadic likelihood method implemented in CoANCESTRY v. 1.0 software (Wang 2007) was used to estimate individual inbreeding coefficients from microsatellite data. This programme also outputs estimates of inbreeding coefficients from an additional likelihood and two moment estimators (Wang 2007). Estimates from different estimators are highly correlated (Spearman $r_s$ average 0.77 for all pairwise combinations of estimators; Wang 2007). To further assess the informativeness of the markers and the reliability of the inbreeding estimates of our dataset, we conducted two sets of simulations. First, we simulated a dataset composed of 100 individuals with the same marker information as the one used in this study (i.e. with the same allele frequency, missing genotype rate and error rate for each of the 19 microsatellite loci). The simulated inbreeding coefficients of the 100 individuals vary from 0 to 1 with an increment of 0.05, and the proportion of individuals in each inbreeding category is the same. The correlation coefficient between the simulated and estimated (by triadic likelihood method) inbreeding coefficients is, on average, 0.88 across 42 replicates. This method has the advantage of using a similar amount of marker information to the original dataset in estimating inbreeding coefficients.

Second, we used a previously designed test by Balloux et al. (2004). The 19 markers were divided at random into two sets, which were used independently to estimate individual inbreeding coefficients. The correlation coefficients between two sets of estimates were then calculated. The simulation was replicated 100 000 times. In each replicate, 10 individuals were drawn (without replacement) at random from the original 230 individuals sampled. Similarly, nine markers were drawn (without replacement) at random from the original set of 19 markers to constitute subset 1 and the remaining 10 markers constituted subset 2. The inbreeding coefficients for each of the sampled 10 individuals were estimated using each of the two subsets of markers, using the allele frequencies calculated from the 230 individuals. A correlation was calculated between inbreeding estimates for each individual obtained from the two subsets of markers. The correlation coefficient thus obtained was 0.26. The correlation between true and simulated inbreeding estimates from the first method and the two sets of inbreeding estimates from the second method confirms that the set of markers used for this study is informative to produce reasonably accurate inbreeding estimates.

(d) Offspring survival

All individuals produced in the September to February breeding season of 2006–2007 were sampled and recorded as dead or alive from the point of laying (if embryo developed) through to fledging. To detect inbreeding depression for survival, all individuals were categorized into one of two stages:

—Embryo death: non-viable eggs by the 14th day of incubation ($n = 37$) when they are expected to hatch.
—Nestling death: chicks that hatched yet died in nest by the 24th day of the nesting period ($n = 52$) when they are expected to fledge.

Embryo deaths were indentified only in eggs with obvious signs of embryonic development (approximately stage 14, where the formation of the eyes, head and tail bud are clearly visible; Hamburger & Hamilton 1951). Eggs with no sign of embryo development were judged unfertile and discarded from further analysis. However, these may include fertilized and very early embryo deaths. An additional consideration in using visually identified failed embryos was to obtain enough reliable DNA for amplification. Even if early death embryos could be identified, there is a risk of contamination from parental DNA in the vitelline membrane (Arnold et al. 2003).

Each category was analysed separately using a generalized linear mixed model with a binomial response variable (dead/alive), and inbreeding (continuous variable), sex, clutch size at laying point, maternal inbreeding level and maternal age as potential explanatory variables. Mother identification was set as the random factor to control for nest site and other potential maternal effects. Maternal inbreeding level was produced using CoANCESTRY (Wang 2007) from known maternal genotypes and those reconstructed using the female’s offspring genotypes in COLONY v. 2.0 (Wang & Santure 2009). Model selection for all analyses was performed using Akaike’s information criterion (AIC; Burnham & Anderson 1998) in the R statistical programming environment (R Development Core Team 2007).

(e) Lethal equivalents

To quantify the number and impact of deleterious alleles on survivorship owing to inbreeding, we calculated the number of lethal equivalents (following Morton et al. 1956). This allowed us to provide a standardized measure of inbreeding load to facilitate comparisons across studies (Keller et al. 2002). The probability of survival across all stages of development from embryo to fledgling was modelled using the R statistical programming environment (R Development Core Team 2007). The analysis was structured after Armstrong & Cassey (2007) by fitting the best model of survival that included individual inbreeding coefficient as covariate and the logit link function used to fit the model $\ln(S_i) = \ln(S_0) + Bf$. The parameter $B$ is the number of lethal equivalents, $S$ is survival and $f$ the level of inbreeding. As most studies have used the original method described by Morton et al. (1956) to estimate lethal equivalents, we also included it.

3. RESULTS

(a) Effect of inbreeding on offspring survival

A total of 98 clutches (387 eggs) were laid by 66 females in the breeding season. The average clutch size was $4.11 \pm 0.04$. Of these, 250 offspring hatched successfully and 137 eggs remained unhatched. Embryo development was found in 39 eggs (28.5% of unhatched eggs); 98 eggs showed no sign of development (71.5% of unhatched eggs) and 54 of these showed signs of degradation (55.1% of undeveloped eggs). Of the hatched offspring, 195 were sampled and successfully genotyped (78% of hatched eggs), 53 were not recovered or not blood-sampled (21.2% of hatched eggs), and two failed to amplify at the PCR stage (0.8% of hatched eggs).

The distribution of inbreeding coefficients across all individuals in the survival analysis is shown in figure 1. The average inbreeding level was $f = 0.08 \pm 0.009$, with 41 individuals showing inbreeding levels higher than $f = 0.125$. The highest-ranked models at both stages of development gave clear support for inbreeding and sex
with least parameters including inbreeding, we found that $B = 1.03$ (CI, $+0.37$ and $+1.7$) for males and $B = 0.6$ (CI, $-0.96$ and $+2.08$) for females. However, these data omit information on infertility and early embryo death. Eggs were removed after 14 days of being laid and many may have decomposed, which may have caused degradation of the genomic DNA, making it impossible to distinguish between infertility and early embryo death. This may lead to the underestimation of $B$. The number of lethal equivalents estimated using the modelling method is moderate.

As most studies to date have historically used the regression method developed by Morton et al. (1956), we have also adopted it in this study. However, this method does not account for other factors that may interact with inbreeding and is limited when survival is zero (Armstrong & Cassey 2007). We found the lethal equivalents carried by the population until fledged were 6.91 per haploid genome.

4. DISCUSSION

There are four main results of this study: (i) inbreeding depresses survival; (ii) there are more inbred males than females at the embryonic stage of development; (iii) there is male-biased mortality owing to inbreeding; and (iv) hihi show an appreciable genetic load at early stages of development. Case studies that allow the accurate quantification of such strong inbreeding depression in reintroduced wild vertebrate populations are rare (but see examples below). This study adds support to the caution expressed in the conservation genetic literature of the insidious effects inbreeding can have on population fitness (Frankham et al. 2002), especially when using reintroduction management.

A number of hypotheses have been tested to understand the factors that influence survival and the long-term persistence of this species. We have found that an individual’s level of inbreeding is strongly correlated to the probability of persistence. Hatching failure and poor survival can also be influenced by non-genetic environmental variables (Briskie & Mackintosh 2004), such as food supply (including nutrients), disease and parental quality. In a previous correlational study, female age and high ambient temperatures were linked to hatching success in this species (Low & Párt 2009). However, this study found maternal age influenced nestling survival, but not embryo survival (which is a strong component of hatching success). Experimental studies have also reported that supplementation with carotenoids does not enhance hatching success (Ewen et al. 2008), but that carotenoid supplementation and/or removal of ectoparasites enhances nestling survival (Ewen et al. 2009). Given the use of only a single breeding season’s data and that we have no replication over time, we assume environmental variables such as temperature, nutrient availability and ectoparasites were similar for all individuals. This is further justified because the population is provided with supplementary food (20% by mass sugar water) and ectoparasites are controlled as part of ongoing management of the population.

Sexual size dimorphism is thought to account for different mortality rates between the sexes in many taxa, including birds (Rails et al. 1986; Promislow et al. 1986; Promislow et al. 2004).
Hatching failure is a common outcome of inbreeding (Koenig 1982), whereas the hihi population experiences hatching failure levels over three times higher (approx. 35%), strongly indicative of the effect of an inbred genome on embryological development (Briskie & Mackintosh 2004). Many threatened species that have undergone genetic bottlenecks suffer from high levels of hatching failure. For example, in the reintroduced populations of the New Zealand kakapo (Strigops habroptilus) and takahe (Porphyrio mantelli), hatching failure is around 31 and 66 per cent, respectively (Jamieson & Ryan 2000). The bottlenecked, reintroduced island populations of takahe exhibit twice as much hatching failure as the remnant mainland population (Jamieson & Ryan 2000; Briskie & Mackintosh 2004).

Embryo mortality is a strong contributor to hatching failure in this hihi population, and we have shown that embryo survival is correlated with the individual’s level of inbreeding. Clutch size is also an important factor to consider when interpreting inbreeding effects on hihi embryo and nestling survival, with increased survival in larger clutches. This effect has been found previously (Low & Pärt 2009) and is generally considered a good indicator of maternal quality (Mousseau & Fox 1998), suggesting that either genetically less inbred mothers are producing larger clutches or non-genetic quality variation among breeding females is influencing the extent of inbreeding depression in survival. Maternal inbreeding level was not found to influence offspring survival at the embryonic or nestling stage. However, investigating how the genetic quality of mothers influences their fecundity

Table 1. Model selection to assess the effects of inbreeding (f) on offspring survival in the hihi at two stages of development. Maternal ID was included as a random term. ‘Inbreeding’ corresponds to the individual’s f-value, the ‘sex’ factor distinguishes between males and females, ‘mumage’ refers to the maternal age at the point of sampling, ‘clutch’ refers to the clutch size and ‘muminbreeding’ corresponds to the maternal f-value. AIC is Akaike’s information criterion, AICc is the corrected AIC value for small sample sizes, Δi is the difference in AICc value from that of the strongest model, K is the number of parameters in the model, n the sample size and W_i is the Akaike weight. The selected models are in bold (i.e. models with the lowest AIC values).

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</table>

1992), owing to the higher energetic requirements during growth for larger offspring (Anderson et al. 1993). Sexual size dimorphism has previously been found in early post-hatching stages of development in hihi (Ewen et al. 2009). At 21 days of age, males are significantly larger than females in terms of weight, tarsus length and head–bill length, but none of these morphological measures are significantly affected by inbreeding (data not shown). However, other variables such as the endocrine system generate fundamental developmental differences between the sexes, including differential immune system responses (Benito & González-Solís 2007). Therefore, rapid male growth and different endocrine profiles may increase developmental stress in males, making them more vulnerable to effects of inbreeding during growth.

The much lower numbers of highly inbred females at the embryonic stage in our cohort could have arisen, for example, owing to (i) the number of inbred males and females being initially equal, but highly inbred females having perished at a very early embryo stage (pre-testing stage); (ii) more closely related pairs producing more sons than daughters; or (iii) a sampling bias (for example, if the non-viable eggs that could not be retrieved in the field or analysed were more frequently females than males). Which explanation proves correct is an important future consideration requiring targeted research.

Hatching failure is a common outcome of inbreeding in wild populations (Van Noordwijk & Scharloo 1981; Bensch et al. 1994; Keller 1998). In outbred avian populations, hatching failure averages approximately 10 per cent (Koenig 1982), whereas the hihi population experiences hatching failure levels over three times higher (approx. 35%), strongly indicative of the effect of an inbred genome on embryological development (Briskie & Mackintosh 2004). Many threatened species that have undergone genetic bottlenecks suffer from high levels of hatching failure. For example, in the reintroduced populations of the New Zealand kakapo (Strigops habroptilus) and takahe (Porphyrio mantelli), hatching failure is around 31 and 66 per cent, respectively (Jamieson & Ryan 2000). The bottlenecked, reintroduced island populations of takahe exhibit twice as much hatching failure as the remnant mainland population (Jamieson & Ryan 2000; Briskie & Mackintosh 2004).

Embryo mortality is a strong contributor to hatching failure in this hihi population, and we have shown that embryo survival is correlated with the individual’s level of inbreeding. Clutch size is also an important factor to consider when interpreting inbreeding effects on hihi embryo and nestling survival, with increased survival in larger clutches. This effect has been found previously (Low & Pärt 2009) and is generally considered a good indicator of maternal quality (Mousseau & Fox 1998), suggesting that either genetically less inbred mothers are producing larger clutches or non-genetic quality variation among breeding females is influencing the extent of inbreeding depression in survival. Maternal inbreeding level was not found to influence offspring survival at the embryonic or nestling stage. However, investigating how the genetic quality of mothers influences their fecundity
Sex-specific inbreeding depression observed in early life stages suggests an indirect physiological mechanism. Direct phenotypic consequences of inbreeding should not differ between females and males unless key loci are sex-linked (Frankham & Wilcken 2006). If highly inbred females are dying at the very early embryo stage beyond the detection capability of this study, then this may suggest a direct physiological mechanism in which the heterogametic sex is more vulnerable owing to sex-linked recessive genes (Haldane 1922). Sex-specific effects of inbreeding have previously been found in two wild avian populations: the Mandarte Island song sparrow (Melospiza melodia; Keller 1998; Reid et al. 2007) and New Zealand takahe (P. mantelli; Jamieson et al. 2003). Song sparrow (Keller 1998) and takahe (Jamieson 1998) and more widely used and in our study produces a much larger number of lethal equivalents as it does not account for the effects of other interacting variables. Therefore, when comparing with other studies that use this approach, hihi have a moderate to high genetic load (e.g. song sparrow (M. melodia), Keller 1998; collared fly-catcher (Ficedula albicollis), Kruuk et al. 2002; Darwin finches, Keller et al. 2002; great tit (Parus major), Szulkin & Sheldon 2007) and is also higher than most estimates reported for laboratory populations, and lower than those reported for zoo populations (for reviews see Lynch & Walsh 1998; Keller 1998).

The number of lethal equivalents suggests that purging of the genetic load has not yet occurred in this population, in spite of 15 generations of isolation on Tiritiri Matangi, and a restricted remnant population size previously including several bottleneck events (in the remnant population and reintroduction bottleneck). It is possible that a substantial proportion of the inbreeding load for survival in embryo and early developmental stages in hihi is due to many deleterious alleles of minor effects so that inbreeding and selection are ineffective in removing them (Hedrick 1994; Wang et al. 1999).

Genetic rescue is a possible management option for hihi given that the remnant population has reasonable numbers of individuals available to translocate into the Tiritiri Matangi population. In cases where this is not possible (too few individuals or only single populations), there are alternative approaches that could be used to manage inbreeding. In the New Zealand kakapo, for example, individuals are translocated from the breeding population to improve breeding prospects of remaining and less-related individuals (Elliott et al. 2001), and in the New Zealand takahe closely related pairs are separated to allow them to find more suitable partners (Jamieson 2009). Importantly, our results add support to the concerns that inbreeding depression in isolated island avifauna, often also the focus of reintroduction programmes, is more common than previously appreciated (Jamieson 2009).

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