Impact of sexually antagonistic genital morphologies on female reproduction and wild population demography

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Authors' contributions:

Y.T. and M.K. conceived the study. Y.T. performed the experiment of individual fitness. Y.T., T.F., J.Y., and M.K. performed the population genetic and comparative analyses. Y.T. drafted the manuscript and revised it in cooperation with J.Y. and M.K. All authors discussed the results and implications of the study at all stages.

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Abstract

Sexual conflict is a strong driver of evolution. The evolutionary outcomes of sexual conflict can, in turn, influence ecological processes within populations, e.g., demography. However, evidence for the latter hypothesis is scarce, especially in the wild. Here, we show that sexual conflict is associated with demographic processes determining population size in the ground beetle *Carabus insulicola* with elaborate male and female genitalia, based on individual- and population-level analyses. We found that sexually antagonistic selection can operate on the genitalia: longer male genitalia can be beneficial in sperm competition but decrease female reproductive success with increased egg dumping, while longer female genitalia are resistant to this male harassment via decreased egg dumping and increased fertilization rate. As expected from sexually antagonistic coevolution due to sexual conflict, we detected coevolutionary divergence between male and female genital sizes among populations. In parallel with decrease in female reproductive success, more harmful males with longer genitalia and less resistant females with shorter genitalia were related to small effective population sizes. Thus, sexual conflict may promote coevolutionary diversification between sexual traits, and this was associated with a demographic process. Our findings provide an insight into sex-driven eco-evolutionary dynamics in the wild.

Keywords: *Carabus insulicola*, eco-evolutionary dynamics, population size, sexual conflict, sexual selection

The tragedy of the commons occurs when the maximization of an individual's profit prevents a population-level benefit. This is expected as an outcome of evolutionary processes in social and sexual contexts because natural and sexual selection maximize relative fitness, rather than absolute fitness (Rankin et al. 2007). Frequently, female fitness is reduced by male adaptations to female multiple mating, which promotes sexual conflict (Arnqvist and Rowe 2005). Adaptations to promote male persistence to mate and those to improve female resistance against male coercion have been demonstrated to result in further evolutionary outcomes. Antagonistic coevolution between sexes is a possible outcome (Arnqvist and Rowe 2002, Rönn et al. 2007, Perry and Rowe 2011), while the evolution of female fitness tolerance to male mating harm will halt the coevolutionary arms race (Gosden and Svensson 2009, Svensson and Råberg 2010). Decrease in female reproductive rate via sexual conflict may also promote ecological processes such as decrease in population growth rate (Holman and Kokko 2013). Theory predicts that the fitness costs to females due to male harassment may result in decreased population sizes, and even extinction (Kokko and Brooks 2003, Rankin et al. 2011); this is supported by experimental evolution studies in insects (Holland and Rice 1999, Martin and Hosken 2004, Rankin and Arnqvist 2004, Arnqvist and Tuda 2010, Gay et al. 2011). Thus, sexual conflict can play roles in both evolutionary and ecological processes, and may mediate interplay between them, i.e., eco-evolutionary dynamics (Kokko and López-Sepulcre 2007, Hendry 2017).

Although theoretical and experimental evidence for the effects of sexual conflict on population fitness are increasing as discussed above, empirical studies demonstrating the direct effects of sexual conflict on population size in the wild are rare. In an enclosure experiment, a male-biased sex ratio in lizards reduced female survival and fecundity; therefore, a decrease in population size was expected (Le Galliard et al. 2005). In the common bluetail damselfly *Ischnura elegans*, the evolution of a female color polymorphism decreased male harassment of females and increased the productivity, density and effective size of population (Takahashi et al. 2014). Comparative analyses have shown that a proxy for sexual selection is associated with a proxy for population size (threat status) (Morrow and Pitcher 2003, Bro-Jørgensen 2014), but conflicting results have been obtained (Morrow and Fricke 2004). Elucidating the causal relationship between sexual conflict and population size are expected to improve our understanding of eco-evolutionary dynamics, but it has remained largely unexplored, especially in wild populations.

The purpose of this study was to examine whether and how sexual conflict influences demographic process. We focused on the male and female genital structures of insects as agents of sexual conflict. Male genitalia can be pertinent to behavioral and physiological manipulation of the female, and this process can inflict fitness costs to the female (Arnqvist 1989, Crudgington and Siva-Jothy 2000, Hotzy and Arnqvist 2009). Female genitalia can evolve counter-adaptation to the male manipulation and decrease fitness cost incurred because of male manipulation (Arnqvist and Rowe 1995, Rönn et al. 2007). Such an evolutionary tug of war can result in coevolutionary divergence between the male and female genital structures (Arnqvist and Rowe 2002, Rönn et al. 2007, Perry and Rowe 2011). If sexual conflict influences demographic process, it is expected that population size covaries with the coevolutionary dynamics between the male and female genital structures: population size and female reproductive rate will increase when the female counter-adaptation has the upper hand over the male manipulation, and *vice versa*.

To this end, we performed experimental and comparative analyses using wild populations of the ground beetle *Carabus insulicola* (Fig. 1A). Males have a highly sclerotized hook-like structure (copulatory piece, CP) on the intromittent organ (Figs. 1B, D). Females have a membranous pocket attached to the bursa copulatrix (vaginal appendix, VA), which is the counterpart of the CP (Fig. 1C). The CP is rigidly coupled with the VA during copulation (Fig. 1E) (Ishikawa 1987, Takami 2002). The CP is an adaptation for female multiple mating, playing a role in spermatophore deposition and sperm transfer (Takami 2003) as well as displacement of rival spermatophore (Takami 2007, Takami and Sota 2007, Okuzaki and Sota 2014). A morphological mismatch between a long CP and a short VA in ground beetles is expected to result in female fitness costs because the male organ can physically harm the female genitalia. This cost to females is manifested in heterospecific mating with large genital mismatches (Sota and Kubota 1998) and is possible in conspecific mating in species with elongated or spine-like male genitalia (Sota and Kubota 1998, Okuzaki et al. 2012). Physiological manipulation of the female via seminal substances can also be a source of sexual conflict in this group of insects (Takami et al. 2008, Hayashi and Takami 2014), in which the male genitalia may play a role as in the other group of beetles (Crudgington and Siva-Jothy 2000, Hotzy and Arnqvist 2009). The ground beetle species of the subgenus *Ohomopterus* (genus *Carabus*), including *C. insulicola*, exhibit coevolutionary divergence of the CP and VA among species (Ishikawa 1987, Sasabe et al. 2010). Thus, size imbalance between the CP and VA in *C. insulicola* is expected to influence individual- and population-level fitness.

First of all, we examined morphological and genetic differentiation as well as morphological covariation between sexes across nine wild populations of *C. insulicola*, to describe the study system and to confirm an evolutionary outcome of sexual conflict, i.e., coevolutionary diversification in male and female genital sizes. Secondly, we performed a reciprocal cross-population mating experiment using two of nine populations, to detect signatures of sexually antagonistic coevolution between sexes and sexually antagonistic selection operating on male and female genital sizes. Then, we examined the effect of genital size imbalance on population size by comparing nine study populations, to test whether sexual conflict influence demographic process. Based on these results, we discuss evolutionary and ecological processes that mediate covariation among male and female sexual traits and individual- and population-level fitness.

Methods

Study populations and sampling

Carabus insulicola is a flightless ground beetle endemic to Japan. It is univoltine and inhabits grasslands and forest margins, where adults prey on terrestrial invertebrates and larvae depend only on earthworms. Beetles were collected using pitfall traps in nine localities in Miyagi Prefecture, Tohoku District (Figs. 2A, B). These localities were situated at a maximum of 100 km from each other and included six islands and three mainland populations.

Analysis of morphological variation among populations

Sexual conflict and other selective processes are expected to result in trait differentiation among populations and covariation between sexes among populations (Gavrilets 2000, Arnqvist and Rowe 2002, Rönn et al. 2007, Perry and Rowe 2011). To confirm these prerequisites to our hypothesis, we investigated morphological variation and covariation in male and female genitalia among the nine populations (Fig. 2B).

Beetles were collected in 2005 and 2006 and dissected to remove and measure their genitalia and to remove tissues for DNA extraction (described later). The carcasses were then pinned and dried. Body size (length from the apical margin of the labrum to the apices of the elytra) was measured to the nearest 0.01 mm using a digital caliper. CPL and VAL were measured to the nearest 0.001 mm using a digital micrometer (MDC-MJ/PJ; Mitutoyo Corp., Tokyo, Japan).

To examine differentiation in male and female genital morphology between nine populations, a generalized linear model (GLM) with an identity link and a Gaussian distribution was constructed for genital size in each sex, including body size, population, and their interaction as independent variables using R version 3.3.20 (R Core Team 2016). We found no significant interaction between population and male body size ($P = 0.72$), but did find a marginally non-significant interaction between population and female body size ($P = 0.058$). The interaction term was dropped from the male final model, but remained in the female final model. The measurements were log-transformed before the analysis because the estimates of slopes are relevant to genital allometry (Eberhard et al. 1998).

Population genetic analysis

We estimated genetic differentiation among populations using data from microsatellite loci. Total DNA was extracted using the standard phenol-chloroform method. Six microsatellite loci (*Cins*12, *Cins*15, *Cins*25, *Cins*26, *Cins*33, and *Cins*36; Takami and Katada 2001) were genotyped (sample size in Table S1). PCR amplification was carried out using fluorescent-labeled primers with appropriate annealing temperatures (Takami and Katada 2001). Amplified fluorescent fragments were analyzed using an ABI 3130 Genetic Analyzer with GeneScan HD 400 ROX size standard (Applied Biosystems). Band sizes were determined with GeneMapper 4.0 software (Applied Biosystems).

Genetic differentiation in microsatellites is frequently evaluated by F_{ST} (Wright 1921) or R_{ST} (Slatkin 1995), assuming infinite allele model (IAM) and stepwise mutation model (SMM), respectively. The utility of these two methods depends on the type and rate of mutation in a focal locus (Balloux and Lugon-Moulin 2002). SMM did not better fit to our microsatellite data than IAM, because an allele size (i.e., the number of repeats in microsatellites) had no additional information in estimating population differentiation. This was confirmed by non-significant result of allele permutation test using SPaGeDi ver. 1.3 (Hardy and Vekemans 2002) (global R_{ST} vs. F_{ST} for all loci, $P = 0.51$). Thus, F_{ST} was preferred to R_{ST} because R_{ST} has a larger variance (i.e., uncertainty) than F_{ST}. Next, F_{ST} tends to be underestimated in highly polymorphic markers such as microsatellites. Thus, in addition to F_{ST}, genetic differentiation among populations was also evaluated based on Jost's D_{est}, a relative of F_{ST} that is unbiased with respect to genetic diversity (Jost 2008). The statistical significance of F_{ST} and D_{est} was examined by randomization tests with 999 replications using GenAlEx 6.503 (Peakall and Smouse 2006, 2012).

Effective population size (N_{e}) was used as a measure of the size of the populations and estimated by population genetic procedures based on multilocus microsatellite genotypes. A moment estimator of N_{e} based on linkage disequilibrium (LD) was obtained using NeEstimator ver. 1.3 (Peel et al. 2004), and a simulation-based estimator was generated using summary statistics and an approximate Bayesian computation implemented in ONeSAMP ver. 1.2 (Tallmon et al. 2008, available at http://plaza.ufl.edu/surajk95/onesamp/). Briefly, ONeSAMP was developed specifically to microsatellite data involving allele length information. This program calculates eight summary statistics from input data, and samples 50000 simulated populations that match the observed summary statistics. Mean N_{e} value and its 95% credible limits were estimated from these accepted simulated populations. We used upper and lower bounds on the prior for N_{e} of two and 10000, respectively. To evaluate the repeatability of estimates (i.e., the convergence of simulations), we ran two independent simulations for each population.

Experimental analysis of individual fitness

We investigated male and female individual fitness components and their dependence on male and female genital sizes. We conducted two types of intra-population crosses and two reciprocal types of inter-population crosses using two populations, Aobayama (Ao) and Ohshima Is. (Oh) (Fig. 2B), which showed the largest differentiation in VAL. Reciprocal inter-population matings are expected to increase the imbalance between male manipulation and female resistance. If sexual conflict drives sexually antagonistic coevolution, females are expected to be more resistant to manipulation by males of their own population than those of other populations because females had the opportunity for counter-adaptation to males in their own population (Andrés and Arnqvist 2001, Knowles and Markow 2001, Hosken et al. 2002, Nilsson et al. 2002, see also Rowe et al. 2003). Additionally, at the level of variation within populations (or within cross types), if male and female genitalia are sexually antagonistic traits, more elaborate male genitalia are expected to be more manipulative, but more harmful to the female, while more elaborate female genitalia should be more resistant.

Beetles were collected on May 20–22, 2015, the onset of their reproductive season. Live beetles were immediately transferred to the laboratory and kept in an incubator set at 20°C and a 16L8D photoperiod to induce and maintain reproductive activity. A male and a female were arbitrarily chosen from the same or different populations and allowed to mate freely within a plastic jar (13 cm diameter × 10 cm height), in which females were allowed to lay eggs in moistened soil at the bottom of the jar (2 cm depth) (Total, N = 62; Ao female × Ao male, N = 11; Ao × Oh, N = 11; Oh × Ao, N = 17; Oh × Oh, N = 23). This experiment was started on May 23 and continued for 65 days (comparable to reproductive season in the wild). Beetles were fed with minced beef *ad libitum*. They were checked for death and oviposition every other day. The longevity was regarded as a component of individual fitness. Eggs were collected and kept in a small cup with moistened soil to check their development and hatching. Eggs that developed or hatched were regarded as fertilized, and others were treated as unfertilized. Fertilization rate was calculated as the number of fertilized eggs divided by the total number of eggs oviposited. The number of fertilized eggs and fertilization rate were regarded as fitness components of the male and female, while the number of unfertilized eggs was regarded as female fitness loss due to egg dumping. If a male died, the experiment was continued using only the female, and if the female died, the experiment was terminated. After the experiment, beetles were dissected to remove their genitalia. The presence of scars (melanized patches) was determined in the female genital tract. Female genital scars were assumed to be the result of physical interaction with the male genitalia and regarded as an agent of female fitness loss. The carcasses were then pinned and dried, on which body size was measured. Male and female genital sizes (CPL and VAL) were measured to the nearest 0.001 mm using ImageJ (National Institutes of Health, Bethesda, MD, USA) based on digital images obtained using a binocular microscope (EZ4 HD; Leica Microsystems, Wetzlar, Germany).

To explain variation in male and female individual fitness components, six statistical models were constructed. Four of the six were GLMs with the number of fertilized eggs, number of unfertilized eggs, fertilization rate, and presence of a scar in the female genital tract as dependent variables, and CPL, VAL, male body size, female body size, male longevity, female longevity, male population, female population, and the interaction between male and female populations as independent variables. GLMs with a log link and a negative-binomial distribution were constructed for count data using the function glm.nb in R, to control observed overdispersion in egg count data. GLMs with a logit link and a binominal distribution were constructed for binary or proportional data. The remaining two models were parametric survival models with male and female longevities as dependent variables, and CPL, VAL, male body size, female body size, male population, female population, and the interaction between male and female populations as independent variables. Parametric survival models were constructed using the Survival function in R. Weibull, log-normal, Gaussian, exponential, logistic, and log-logistic distributions were used to verify model suitability, with the most appropriate distribution chosen based on the Akaike information criterion corrected for small sample sizes (AICc). Male survival model assuming logistic distribution indicated the smallest AICc, and only female survival model assuming exponential distribution was successfully fitted (Table S2). Thus, logistic and exponential distributions were assumed for male and female survival data. Statistical significance of coefficients was based on likelihood ratio tests.

Comparative analysis

To account for genetic differentiation among populations, phylogenetic generalized linear mixed models (PGLMMs) were constructed using the MCMCglmm package (Hadfield and Nakagawa 2010) in R, based on the nine populations and a covariance matrix of genetic differentiation. We used 1 - D_{est} as an element of the covariance matrix, which corresponds to heterozygote deficiency due to population differentiation (i.e., $1 - F_{\mathrm{ST}} = H_{\mathrm{S}}/H_{\mathrm{T}}$) and ranges between 0 (complete genetic differentiation) and 1 (no differentiation).

To examine coevolutionary diversification between male and female traits, we constructed comparative analysis models for explaining population means of a male trait with those of a female trait as a sole independent variable. Then, we added N_{e} and land type (mainland or island) to the model as covariates.

To examine the impact of sexual conflict on population size, we investigated the effects of male and female genital sizes on N_{e} across the nine populations. Similar to the individual fitness, populations with a long CP and short VA are expected to suffer from decreased population sizes. Multivariate analyses that include male and female traits are expected to determine whether the evolution of more manipulative male genitalia is associated with population size by adjusting the female resistance to manipulation to keep it constant, but it may be difficult to detect such an association based on univariate analyses that include traits of either sex only (Arnqvist and Rowe 2002, Rönn et al. 2007, Perry and Rowe 2011). Based on this consideration, three comparative analysis models were constructed with N_{e} as a dependent variable. The first model included population means for CPL and VAL as independent effects, and the second and third models included either CPL or VAL as an independent effect. Land type (mainland or island) was also included as an independent effect in these models.

Results

Coevolutionary diversification of genitalia among populations

Male and female genital sizes have diverged among populations. CPL and VAL differed significantly among the 9 study populations (Tables 1, S1). Male and female body sizes also differed significantly among populations (Tables 1, S1). Differentiation in genital sizes was significant even after controlling body size differentiation (Table 1). Thus, diversification in CPL and VAL among populations was not a by-product of body size differentiation. Least square means of log CPL and log VAL for each population were calculated from these models and used in the following analyses as relative genital sizes.

Mean genital sizes covaried between sexes across populations. Although the simple covariation between CPL and VAL was not significant (Table 2), the covariation became significant after adjusting for covariates (Table 2). Male and female body sizes also significantly covaried among populations (Table 2). Relative genital sizes did not covary between sexes (Table 2).

Genetic differentiation among populations and effective population size

We detected significant genetic differentiation among 9 populations based on F_{ST} and D_{est} (Table S3), while differentiations between Aobayama and Katsurajima Is., Aobayama and Ohshima Is., Kahoku and Ohshima Is., and Kahoku and Kanan were marginally non-significant ($P = 0.06 - 0.11$).

Our estimates of N_e varied among populations from 12.1 to 411.5 based on the moment estimator, and 32.0 to 654.6 based on the simulation estimator (Table S1). Estimates from two simulation runs were highly correlated ($R^2 = 0.91$, $N = 8$ [one population failed at the second simulation], $P = 0.0055$), and we reported the estimates from the first series of simulations. The N_e obtained based on moment and simulation estimators involved large uncertainties as indicated by broad and sometimes undefined confidence or credible intervals (Table S1). Generally, the performance of N_e estimation depends on sample sizes and historical and geographical settings that the population experiences (Gilbert and Whitlock 2015). Our estimates based on moment and simulation estimators were consistent with each other because they did not differ significantly (paired *t*-test, $t_7 = 1.46$, $P = 0.19$) and were significantly correlated with each other ($r_8 = 0.77$, $P = 0.027$). Thus, to obtain consistent estimates, these two series of estimates were averaged (ranged from 23.1 to 533.1). Only a simulation-based estimate was obtained in Kanan population, and we must be cautious about relatively large uncertainty in this population. The average estimates of N_e suggested a right-tailed distribution, and normality was confirmed after log-transformation (Shapiro–Wilk test, $P = 0.68$).

Effects of genital size on individual fitness

We found evidence for sexual conflict over oviposition and fertilization activities in relation to genital size. We observed more fertilized and unfertilized eggs oviposited by a female in inter-population matings and higher fertilization rate in one of them (Ao female vs. Oh male, Fig. 3) than in intra-population matings, as indicated by the significant interaction terms between male and female populations (Fig. 3, Table 3, marginally non-significant in unfertilized eggs). Twenty-one females oviposited no eggs (5 [45%] in Ao female vs. Ao male, 2 [18%] in Ao vs. Oh, 0 [0%] in Oh vs. Ao, 14 [61%] in Oh vs. Oh), being excluded from the analysis of fertilization rate (Fig. 3, Table 3).

More importantly, longer CP was more manipulative in female oviposition activity and increased egg dumping, and longer VA was more resistant to male manipulation. Females with a short VA oviposited more unfertilized eggs (Fig. 4B) in mating with a male with a long CP (Fig. 4A), and females with a long VA increased fertilization rate (Fig. 4C, Table 3). Note that these effects of genital sizes are consistent across cross types: we can assume common slopes (Fig. 4) because interaction terms between genital sizes and cross type were not significant ($P > 0.05$) and excluded from the statistical models. In addition, male body size was negatively associated with fertilization rate (Table 3).

We did not observe a significant association between the presence of a scar in the female genital tract and genital sizes (Table 3). Additionally, we did not detect significant associations between male and female longevities and genital sizes (Table 4), probably owing in part to the high survival rates in the experimental period (male, 87.1%; female, 96.8%).

Effects of genital size on population size

As expected, a small population size was related to coupling of more manipulative males and less resistant females (Fig. 5). A longer CP and shorter VA were related to a decrease in N_e (Table 5). N_e was also influenced by environmental conditions; island populations tended to be smaller. Male or female genital size did not explain N_e solely (Table 5). Analysis based on relative genital sizes provided similar results, but the effect of CPL became non-significant (Table 5). Note that absolute rather than relative sizes may be more relevant to physical interaction between male and female genitalia (Fig. 1E). PGLMM and ordinary GLM analyses provided same conclusions (Table 5).

Discussion

Sexual selection and sexual conflict are proposed to be major evolutionary forces driving the diversification of genital morphology (Arnqvist 1998, Eberhard 2010). Our results constitute a novel case supporting this hypothesis. Females were more likely to be stimulated in their oviposition and fertilization activities by unfamiliar males. This observation is concordant with the prediction of sexually antagonistic coevolution (Andrés and Arnqvist 2001, Nilsson et al. 2002). However, we used only two populations in this experiment, and it is unclear whether the observed patterns are represented in other pairs of populations. Thus, we need to interpret the results of population crosses cautiously, and the increased number of experimental populations and crosses can refine the following discussion. The fertilization rate of inter-population crosses was higher than that of intra-population crosses (Table 3), specifically in Ao female (Fig. 3). Increase in fertilization rate can be beneficial to females and might be difficult to be linked to female fitness loss due to male manipulation. However, this pattern may be explained by assuming female choice for unfamiliar males in addition to sexually antagonistic coevolution. First, relatively greater increase in fertilization rate in Ao females mated with Oh males than in Oh females with Ao males may be due to difference in their genital sizes. Since Ao females had longer VA than Oh females (Table 1, Fig. 5), Ao females may be more resistant to manipulation by unfamiliar males than Oh females. Since male genital sizes were similar Ao and Oh populations (Table 1, Fig. 5), variation in male seminal substances may also contribute to this process as reported in other groups of insects (Brown and Eady 2001, Hosken et al. 2002, Nilsson et al. 2002, Yamane 2013). Second, female choice of unfamiliar males can result in uniform increase in fertilization rate in inter-population crosses (Lüpold et al. 2013). This effect may hide a possible decrease in fertilization rate in Oh females (having shorter VA and assumed to be more vulnerable to male manipulation) that mated with Ao males. It is still unclear whether female choice plays a role in the evolution of sexual traits within wild populations of *C. insulicola*, warranting further study.

In contrast to the patterns observed between cross types, the patterns within cross types showed clearer evidence for sexual conflict. Although the mechanism is unknown, CP and VA may be pertinent to the manipulation of oviposition and fertilization. A longer CP increased the number of unfertilized eggs oviposited by a female mate (Fig. 4A), but does not affect male's own fertilization success (Table 3). This process may allow a male to decrease offspring sired by following rival males when a female mates with multiple males, possibly increasing his relative, not absolute, fitness. Consequently, this process can produce sexual selection for longer CP. Sexual selection for CP has also been suggested in other contexts: CP plays a role in displacement of rival spermatophore (Takami 2007, Okuzaki and Sota 2014), and repeated elongation of CP in related species covaries with the proxies of sperm competition (Takami and Sota 2007). On the other hand, females can incur a fitness cost via egg dumping if mated with a male having a long CP. A longer VA minimized this cost by decreasing egg dumping and increasing the fertilization rate (Figs. 4B, C). Thus, a longer VA can be more resistant to male manipulation and is expected to be favored as a counter-adaptation. The mechanism of this female resistance is also unknown, but some inference can be made based on genital mechanics. Given the rigid coupling of the CP and VA during copulation (Fig. 1E), the tip of the CP is more likely to reach the bottom of a relatively short VA, producing a greater force that deforms the female genital tract (see Fig. 1B for male genital movement). A long VA may mitigate such a male genital function. These selection processes operating on male and female genitalia may drive sexually antagonistic coevolution and result in the observed coevolutionary diversification in the CP and VA lengths. Covariation between CP and VA lengths were relatively weak and only significant after controlling covariates, suggesting that processes other than sexually antagonistic coevolution may also play a role (Gosden and Svensson 2009, Svensson and Råberg 2010).

The causal relationship between sexual conflict and population size can be bidirectional: sexual conflict may influence the properties of a population as we hypothesized, while sexual conflict may also be influenced by the properties of a population (Eldakar et al. 2009). Theory predicts that sexual conflict operates more strongly in large populations owing to the high density of competing individuals and high levels of standing genetic variation (Gavrilets 2000). Experimental evolution studies supported this (Martin and Hosken 2003, Gay et al. 2011). However, wild populations of a water strider show stronger sexual selection and greater evolutionary response with decreasing population density (Arnqvist 1992). Since comparative analysis between sexual traits and population size is correlational, distinguishing the directions of causality may be difficult. Thus, although we found an association between genital size imbalance and population size as expected from the hypothesis that sexual conflict decreases population size, it may not contradict a hypothesis assuming the opposite causality. The result of our comparative analysis can provide evidence for distinguishing between these two causalities: the trajectory of coevolution between the male and female genital sizes is not parallel with, but perpendicular to, the variation in population size (Fig. 5). This is difficult to be predicted by the hypothesis that sexually antagonistic coevolution is promoted in large populations, but is a principal prediction of the hypothesis that sexual conflict decreases population size.

We assumed that N_e is a good estimate of the census population size, and this is confirmed in a related study (Takahashi et al. 2014). However, this assumption could be violated in some situations. In addition, although we detected a statistically significant association (Fig. 5), sample size (n = 9) was relatively small. Thus, we need to interpret our results cautiously. Our comparative analysis provided an implication about the determinant of N_e in sexual contexts. Sexual conflict and sexual selection can bias reproductive contribution of individuals, where winners (i.e., males winning male-male competition, males chosen by females, females mated with good or sexy males, manipulative males and resistant females in sexual conflict, etc.) tend to monopolize reproductive contribution to the next generation. Thus, N_e is influenced by the mating system and decreases as the reproductive skew among individuals and/or between sexes increases (Nunney 1993), and this can be independent of census size. Reproductive skew among individuals can occur in two ways in sexual contexts: a decrease in reproductive females relative to reproductive males, and *vice versa*. Here, we focused on the former process to examine whether sexual conflict decreased female reproductive output and impacted population size. By contrast, we did not explicitly evaluate the latter process, i.e., a decrease in male reproductive success. Sexual conflict and other sexual selection processes could bias male reproductive success toward a subset of male individuals, resulting in a biased operational sex ratio toward females and a decrease in N_e. However, the results of our comparative analysis suggest that this was not the case in this study. Operational sex ratio is expected to be female-biased when female resistance traits are relatively more elaborate than male manipulative traits, because female threshold of accepting males is relatively higher. N_e is expected to decrease in such a situation via a decrease in male individuals contributing to reproduction. In contrast to this expectation, our results indicated that N_e was relatively large in this situation (upper left part in Fig. 5). Intensive sperm competition can also increase reproductive skew among males, but there was no association between male genital size *per se* (an indicator of the degree of sperm competition; Takami and Sota 2007) and N_e. Thus, we can assume that variation in N_e observed in this study was not largely influenced by a decrease in reproductive males.

In addition to sexual conflict, other ecological factors may influence population size. We found that N_e was low on islands, suggesting that resource limitation and/or colonization process with founder effect and random genetic drift may influence N_e. In addition, the impact of sexual conflict on population size may be manifested in challenging environments, such as islands (Kokko and López-Sepulcre 2007). The presence of related species may influence female fitness through reproductive interference (Kyogoku and Sota 2017, Gomez-Llano et al. 2018). The mainland populations of *C. insulicola* coexist with a congeneric small-sized species *C. albrechti*, but it is difficult to explain decreased population size in islands where the related species is absent. *C. albrechti* is unlikely to result in reproductive interference to *C. insulicola* because of difficulty in heterospecific mating. Reduced predator fauna in islands may influence the ecology and evolution of prey species (Runemark et al. 2014), but predator dropout expected in islands can not explain observed decreases in island population size. Little is known about variation in predator fauna in our study area.

In conclusion, imbalance between the male and female genital sizes was associated with female reproductive rate and population size in the ground beetle *C. insulicola*. These results are concordant with the hypothesis that sexual conflict affect population size, but further examination is necessary for elucidating factors lying between the process determining female reproductive rate and that determining population size. Ground beetle species with elaborate male and female genital morphologies provide a novel system that sheds light on sexual conflict and ecological and evolutionary processes in the wild.

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Table 1 General linear models explaining variation in genital and body sizes of the male and female among 9 populations.

	β ± s.e.	Statistic	P
Model explaining log male genital size			
~Population		$F_{8,175} = 20.95$	**< 0.0001**
~Population + log body size			
Population		$F_{8,174} = 13.27$	**< 0.0001**
log body size	0.17 ± 0.06	$F_{1,174} = 7.33$	**0.0075**
Model explaining log male body size			
~Population		$F_{8,177} = 7.96$	**< 0.0001**
Model explaining log female genital size			
~Population		$F_{8,198} = 12.39$	**< 0.0001**
~Population + log body size + population* log body size			
Population		$F_{8,188} = 8.86$	**< 0.0001**
log body size	0.21 ± 0.20	$F_{1,188} = 1.02$	0.31
Population*log body size		$F_{81,88} = 1.93$	0.058
Model explaining log female body size			
~Population		$F_{8,199} = 11.72$	**< 0.0001**

Table 2 Phylogenetic generalized linear mixed models for detecting covariation between male and female genital sizes and between male and female body sizes across 9 populations.

	β	95% credible limit
Model explaining male genital size		
~female genital size	0.17	-0.21, 0.59
~female genital size + N_e + island/mainland		
Female genital size	0.46	**0.08, 0.85**
N_e	-0.09	**-0.16, -0.01**
Island/mainland	-0.11	-0.27, 0.03
Model explaining male body size		
~female body size	0.72	**0.40, 1.06**
Model explaining relative male genital size		
~relative female genital size + N_e + island/mainland		
Relative female genital size	0.25	-0.13, 0.60
N_e	-0.02	-0.04, 0.01
Island/mainland	-0.01	-0.06, 0.04

Table 3 Generalized linear models explaining variation in male and female fitness components in experimental matings using two populations. Male and female populations indicate difference of Ohshima Is. from Aobayama.

	Number of eggs fertilized			Number of eggs unfertilized			Fertilization rate			Female genital scar		
	Estimate ± SE	χ^2_1	*P*	Estimate ± SE	χ^2_1	*P*	Estimate ± SE	χ^2_1	*P*	Estimate ± SE	χ^2_1	*P*
Male genital size	2.27 ± 1.90	1.48	0.22	5.40 ± 2.31	5.38	**0.020**	-1.84 ± 1.38	1.82	0.18	4.60 ± 3.82	1.56	0.21
Female genital size	-1.36 ± 0.91	1.62	0.20	-2.67 ± 1.08	5.36	**0.020**	1.77 ± 0.77	5.35	**0.021**	-0.43 ± 1.76	0.06	0.81
Male body size	0.25 ± 0.21	1.24	0.27	0.10 ± 0.25	0.17	0.68	-0.37 ± 0.16	5.62	**0.018**	0.22 ± 0.40	0.30	0.59
Female body size	-0.11 ± 0.19	0.27	0.61	-0.03 ± 0.23	0.01	0.92	-0.16 ± 0.16	1.05	0.31	0.01 ± 0.36	0.00	0.97
Male longevity	-0.00 ± 0.02	0.01	0.91	-0.03 ± 0.02	1.53	0.22	0.02 ± 0.01	2.84	0.092	-0.04 ± 0.04	1.19	0.28
Female longevity	0.02 ± 0.03	0.53	0.47	0.02 ± 0.03	0.28	0.60	0.00 ± 0.03	0.00	0.95	-0.76 ± 76.86	3.10	0.078
Male population	1.57 ± 0.55	7.11	**0.0077**	0.42 ± 0.64	0.33	0.57	0.69 ± 0.47	2.10	0.15	-0.91 ± 1.05	0.76	0.38
Female population	1.27 ± 0.48	5.78	**0.016**	0.81 ± 0.56	1.70	0.19	0.40 ± 0.41	0.90	0.34	-0.41 ± 0.86	0.22	0.64
Male population x female population	-3.23 ± 0.68	17.19	**< 0.0001**	-1.53 ± 0.79	3.55	0.060	-1.56 ± 0.53	8.32	**0.0039**	0.39 ± 1.30	0.09	0.76

Table 4 Parametric survival models explaining variation in male and female longevities in experimental matings using two populations. Male and female populations indicate difference of Ohshima Is. from Aobayama.

	Female longevity			Male longevity		
	Estimate ± SE	χ^2_1	*P*	Estimate ± SE	χ^2_1	*P*
Male genital size	-0.01 ± 1.52	0.00	0.99	0.08 ± 0.90	0.01	0.93
Female genital size	-0.08 ± 0.74	0.01	0.91	0.16 ± 0.45	0.13	0.72
Male body size	0.01 ± 0.17	0.00	0.97	0.00 ± 0.10	0.00	0.99
Female body size	0.01 ± 0.15	0.01	0.94	-0.01 ± 0.09	0.01	0.92
Male population	0.08 ± 0.46	0.03	0.87	-0.12 ± 0.28	0.18	0.67
Female population	0.00 ± 0.40	0.00	0.99	-0.01 ± 0.24	0.00	0.97
Male population x female population	-0.07 ± 0.58	0.01	0.91	0.12 ± 0.34	0.12	0.72

Table 5 General linear models (GLM) and phylogenetic generalized linear mixed models (PGLMM) explaining variation in effective population size (N_e) among 9 populations.

	GLM			PGLMM	
	β ± s.e.	Statistic	*P*	β	95% credible limit
Model including both sexes		$F_{3,5} = 8.04$	**0.023**		
Male genital size	-7.18 ± 2.38	$F_{1,5} = 9.10$	**0.030**	-7.14	**-12.76, -0.91**
Female genital size	4.42 ± 1.17	$F_{1,5} = 4.60$	**0.013**	4.41	**1.46, 7.33**
Island/mainland	-0.61 ± 0.21	$F_{1,5} = 8.44$	**0.034**	-1.22	**-2.33, -0.12**
Model including both sexes		$F_{3,5} = 3.79$	0.093		
Relative male genital size	-22.60 ± 15.02	$F_{1,5} = 2.26$	0.19	-26.85	-70.18, 12.08
Relative female genital size	12.67 ± 4.66	$F_{1,5} = 7.39$	**0.042**	13.47	**2.58, 24.81**
Island/mainland	-1.18 ± 0.57	$F_{1,5} = 4.27$	0.094	-1.15	-2.55, 0.17
Model including male only		$F_{2,6} = 1.53$	0.29		
Male genital size	-4.27 ± 4.04	$F_{1,6} = 1.12$	0.33	-4.18	-13.52, 6.55
Island/mainland	-0.43 ± 0.37	$F_{1,6} = 1.17$	0.29	-0.86	-2.68, 0.74
Model including female only		$F_{2,6} = 3.19$	0.11		
Female genital size	3.28 ± 1.69	$F_{1,6} = 3.74$	0.10	3.23	-0.63, 7.49
Island/mainland	-0.67 ± 0.32	$F_{1,6} = 4.38$	0.08	-1.34	-2.72, 0.14

Table S1 Morphological and ecological characteristics of nine study populations of *Carabus insulicola*.

Population	Land type	Sample size (male, female, genetic)	Male body size (MBL, mm)	Female body size (FBL, mm)	Male genital size (CPL, mm)	Female genital size (VAL, mm)	N_e based on LD [95% confidence limit]	N_e by ONeSAMP [95% credible limit]
Aobayama	mainland	30, 30, 54	27.75 ± 0.94	29.84 ± 0.97	3.479 ± 0.106	3.237 ± 0.171	411.5 [119.5, -]	654.6 [215.6, 6528.7]
Kahoku	mainland	9, 25, 34	27.30 ± 1.31	28.80 ± 0.91	3.398 ± 0.128	2.796 ± 0.323	271.7 [71.2, -]	107.1 [49.5, 424.2]
Kanan	mainland	4, 15, 19	27.33 ± 0.47	29.47 ± 1.03	3.425 ± 0.059	2.833 ± 0.149	- [53.4, -]	45.4 [22.6, 147.1]
Ajishima Is.	island	30, 30, 56	27.54 ± 0.89	28.66 ± 1.09	3.359 ± 0.093	3.106 ± 0.178	98.0 [53.0, 333.1]	124.4 [55.9, 439.4]
Katsurajima Is.	island	31, 27, 50	28.64 ± 0.97	30.73 ± 0.74	3.585 ± 0.090	3.216 ± 0.274	27.0 [19.9, 38.6]	32.0 [17.5, 91.4]
Nonoshima Is.	island	25, 33, 55	27.99 ± 1.23	29.62 ± 0.95	3.455 ± 0.094	3.061 ± 0.299	33.1 [23.4, 50.1]	51.8 [24.8, 227.7]
Ohshima Is.	island	10, 11, 21	27.53 ± 0.93	29.12 ± 1.46	3.482 ± 0.066	2.736 ± 0.241	12.1 [8.8, 17.6]	34.0 [19.8, 110.1]
Sabusawajima Is.	island	17, 10, 26	28.76 ± 0.69	30.40 ± 0.66	3.592 ± 0.112	3.171 ± 0.171	20.9 [13.3, 38.2]	40.4 [19.5, 138.7]
Tashirojima Is.	island	30, 30, 53	27.08 ± 0.83	28.80 ± 1.22	3.331 ± 0.099	3.061 ± 0.158	123.5 [68.3, 415.4]	233.2 [85.5, 941.1]

MBL, male body length; FBL, female body length; CPL, male copulatory piece length; VAL, female vaginal appendix length; LD, linkage disequilibrium.

Table S2 Comparison of survival models assuming different distributions.

Distribution	AICc of male survival model	AICc of female survival model
Weibull	-	-
log-normal	298.47	-
Gaussian	278.85	-
exponential	586.86	641.21
logistic	174.03	-
log-logistic	193.63	-

- failed to fit the model

Table S3 Genetic differentiation between populations as evaluated by F_{st} (lower triangle) and D_{est} (upper triangle). Boldface indicates significance ($P < 0.05$).

	Aobayama	Kahoku	Kanan	Ajishima Is.	Katsurajima Is.	Nonoshima Is.	Ohshima Is.	Sabusawajima Is.	Tashirojima Is.
Aobayama	–	**0.032**	**0.026**	**0.145**	**0.046**	**0.130**	0.017	**0.146**	**0.087**
Kahoku	**0.016**	–	0.020	**0.129**	**0.053**	**0.151**	0.021	**0.127**	**0.103**
Kanan	**0.017**	**0.017**	–	**0.064**	**0.058**	**0.121**	**0.065**	**0.134**	**0.152**
Ajishima Is.	**0.046**	**0.044**	**0.028**	–	**0.193**	**0.202**	**0.211**	**0.258**	**0.285**
Katsurajima Is.	0.021	**0.024**	**0.029**	**0.066**	–	**0.104**	**0.026**	**0.089**	**0.086**
Nonoshima Is.	**0.051**	**0.059**	**0.052**	**0.075**	**0.046**	–	**0.158**	**0.136**	**0.288**
Ohshima Is.	0.014	0.017	**0.033**	**0.069**	**0.019**	**0.065**	–	**0.085**	**0.052**
Sabusawajima Is.	**0.064**	**0.059**	**0.064**	**0.105**	**0.047**	**0.072**	**0.047**	–	**0.222**
Tashirojima Is.	**0.031**	**0.038**	**0.054**	**0.088**	**0.034**	**0.106**	**0.025**	**0.095**	–

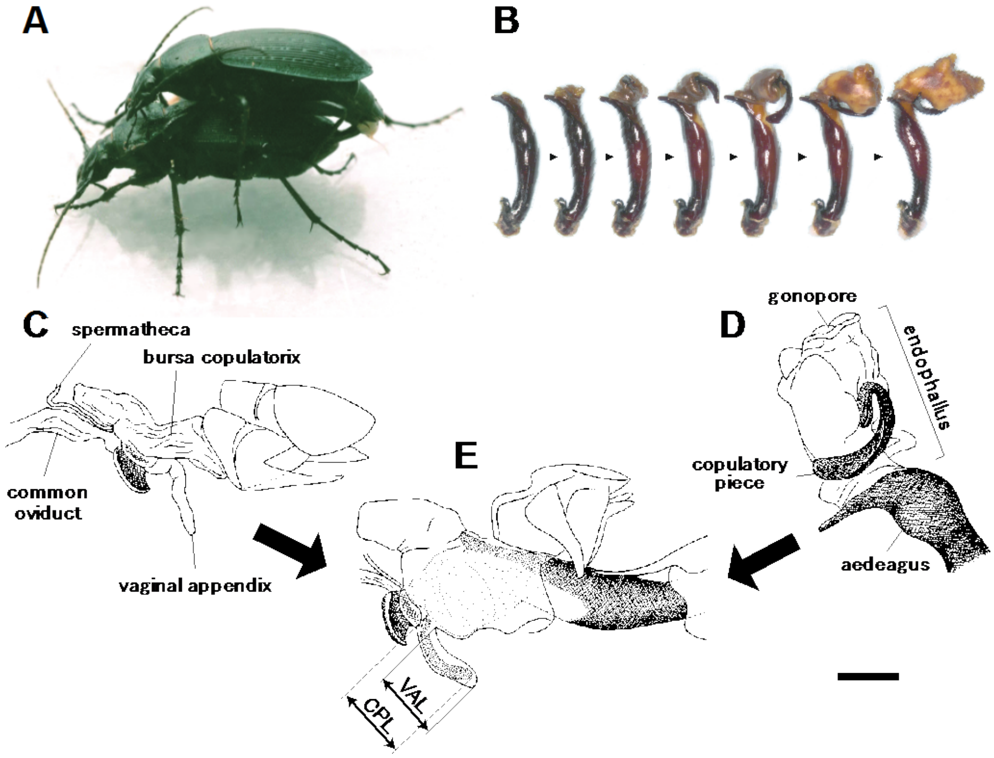

Figure 1 Genital mechanics of *Carabus insulicola*. (A) Male mounting a female *in copula*. (B) The endophallus everting from the aedeagus. (C) The female genitalia include a membranous pocket (vaginal appendix, VA). (D) The male genitalia include a sclerotized hook (copulatory piece, CP). (E) Genital coupling involves insertion of the CP into the VA. CPL, copulatory piece length; VAL, vaginal appendix length. Scale bar, 2 mm for (C), (D) and (E).

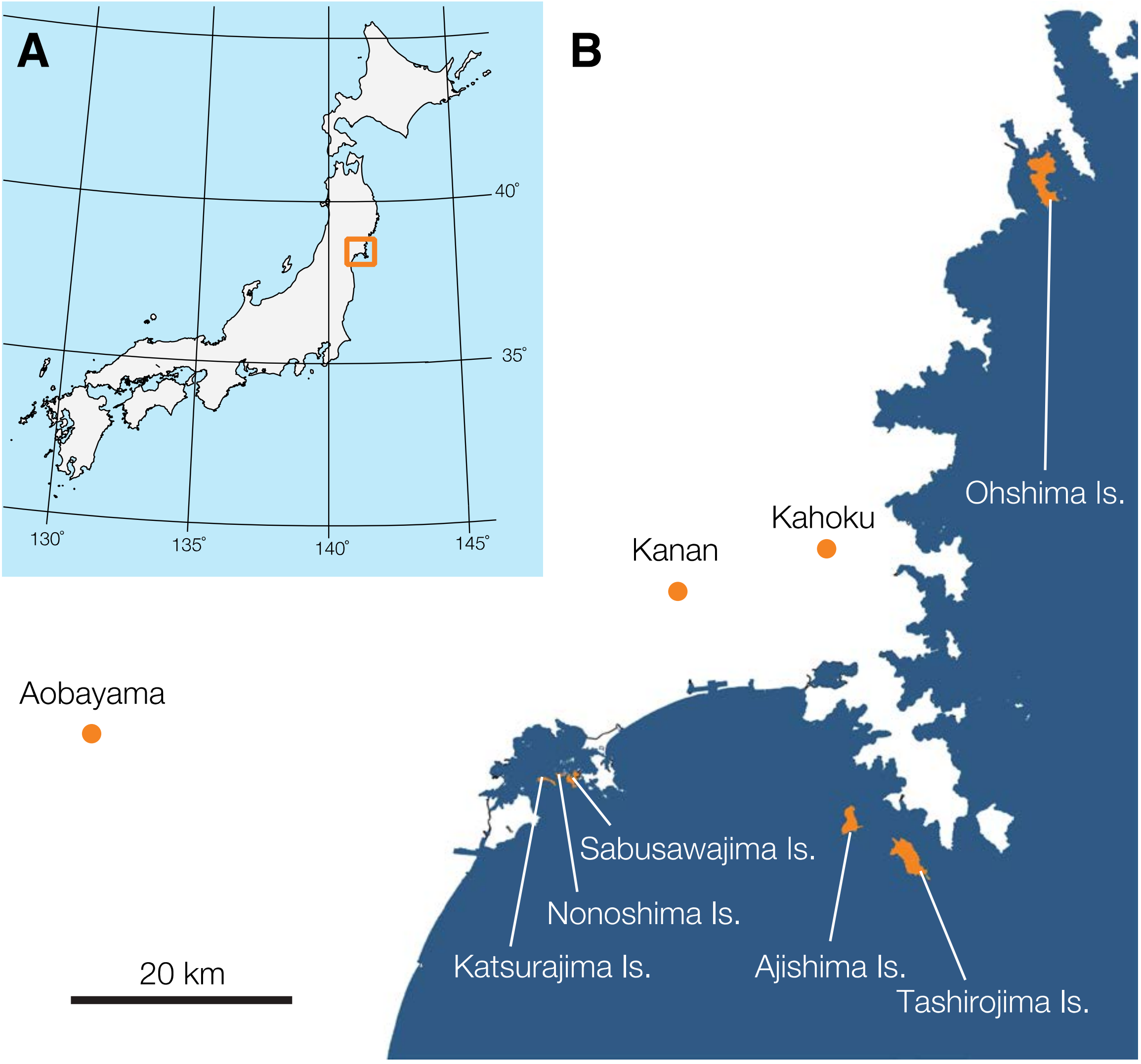

Figure 2 Study populations of *Carabus insulicola*. (A) Map of Japan showing the study area (orange rectangle). (B) Localities of the study populations, including three from the mainland and six from islands.

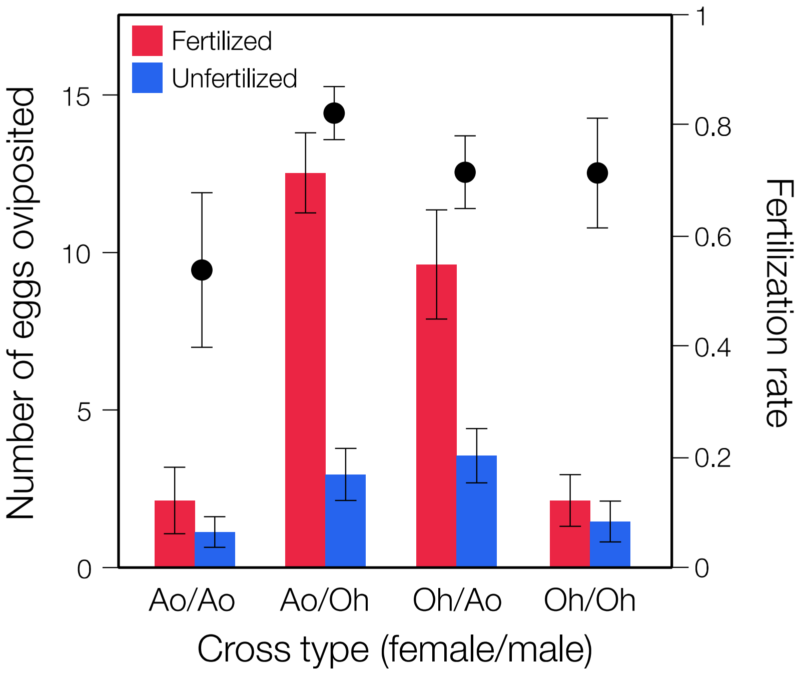

Figure 3 Numbers of fertilized (red bar) and unfertilized (blue bar) eggs oviposited in population crosses and fertilization rates (female/male: Ao/Ao, n = 11; Ao/Oh, n = 11; Oh/Ao, n = 17; Oh/Oh, n = 23). Mean ± 1 s.e.m is shown. The numbers of eggs and fertilization rate increased in interpopulation crosses (see Table 3 in detail).

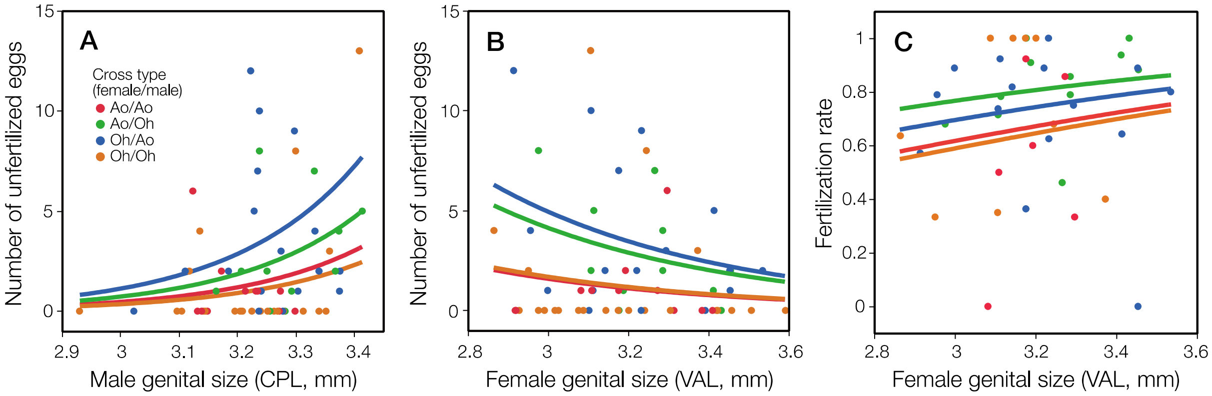

Figure 4 Effect of male or female genital size on the number of unfertilized eggs and fertilization rate. (A) Male genital size vs. the number of unfertilized eggs. (B) Female genital size vs. the number of unfertilized eggs. (C) Female genital size vs. fertilization rate. Regression curves are based on GLMs including male or female genital size and cross type as independent effects. See Table 3 for detailed interpretation based on full models.

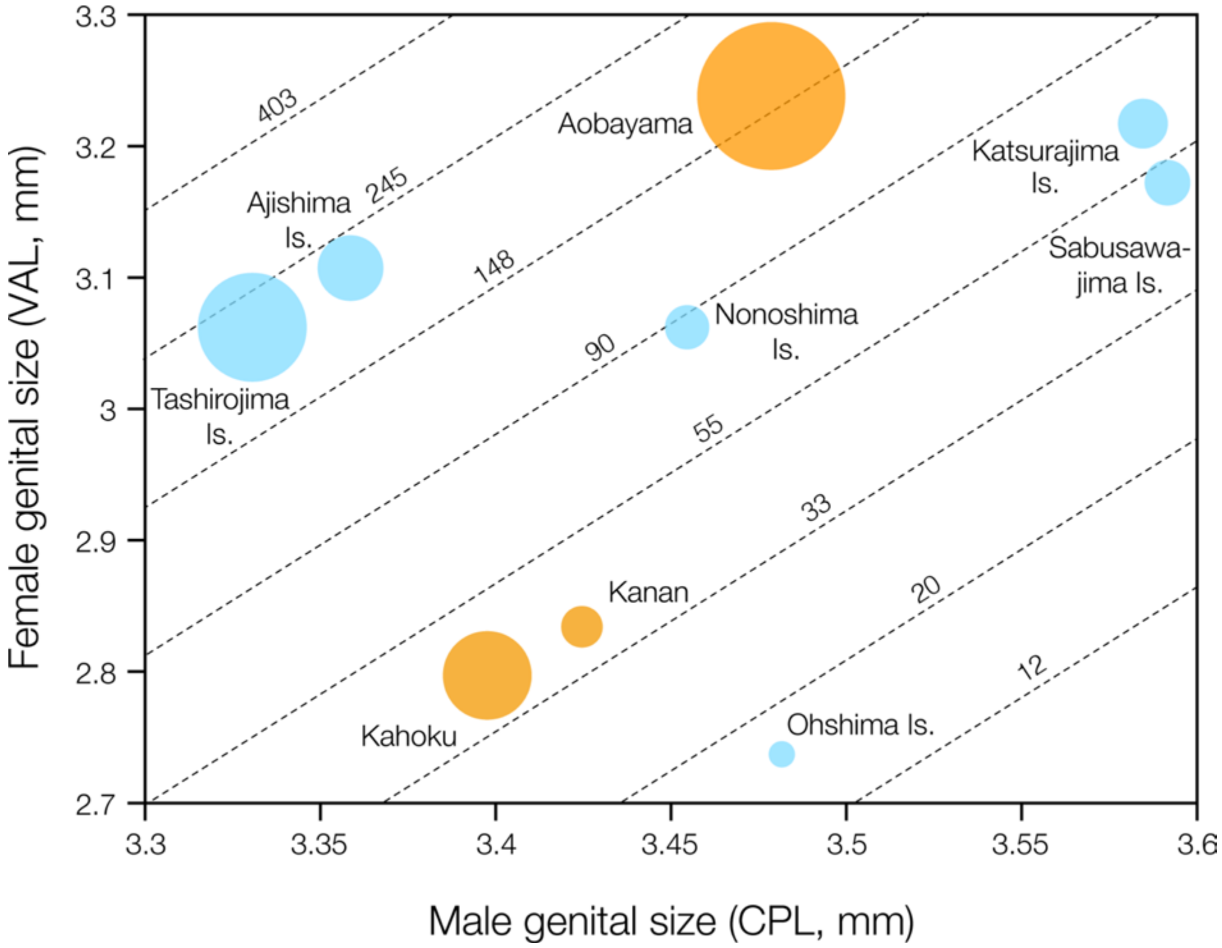

Figure 5 Relationships among male and female genital sizes and population size estimated by a PGLMM model. Male and female genital sizes refer to population means (n = 9). The size of dots refers to averaged N_e obtained from two genetic estimates. Male genital size was negatively, and female genital size was positively associated with N_e. Mainland populations (orange) had larger N_e than island populations (turquoise). Contours and attached numbers represent estimated N_e for island populations from male and female genital sizes. Mainland populations had significantly larger N_e (see Table 5).