Diet dependent sex ratios in *Tigriopus californicus*: Evidence for environmental sex determination in a system with polygenic sex determination

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Abstract

By controlling the inheritance of sex, the sex determination mechanism constrains sex allocation strategies and sex ratio adaptation; however, sex ratio selection also influences the evolution of sex determination mechanisms. Much of the sex determination literature focuses on how sex determination mechanisms transition between genetic and environmental factors (*i.e*. GSD *vs*. ESD), and if genetic sex factors are involved, how many (*e.g*. chromosomal *vs* polygenic systems). The study of sex allocation largely focuses on deviations in sex ratio from a theoretically 1:1 evolutionarily stable strategy, such as when sex ratios reflect 'cost' differences between the sexes. *Tigriopus californicus* is a tidepool copepod with polygenic sex determination, and shows wide variability in sex ratios in the field and lab that cannot be explained by genetic and stochastic processes alone, which suggests that an environmental variable might influence sex ratio. Females and their offspring were fed diets of different nutritional quality in a crossed design, and the sex ratio of each clutch was recorded for up to 8 clutches from a given female: this design allowed the influence of female diet *vs*. that of her offspring to be distinguished. The clutch sex ratio changed over the laying order according to the offspring's diet, which is evidence for environmental sex determination in this species. Sex ratio also showed the influence of maternal diet, consistent with sex allocation theory. While dietary carotenoids showed no association with sex ratio or clutch size, long chain polyunsaturated fatty acids (particularly EPA and DHA) were implicated as the agent of sex ratio effect, providing a direction for future studies. The situation of *T. californicus* at the intersection of major themes in sex evolution makes this system an ideal model for selection studies.

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Dedication

For myself.

Chapter 1: Sex and Sex Determination Mechanisms

Reproduction is fundamental to the continuance of species: it may be achieved sexually, involving two parental genotypes, or asexually, involving a single parental genotype (*e.g.* self-fertilization, parthenogenesis; Schwander *et al.* 2014). Crossover during meiosis is one way genetic recombination is achieved within sexual reproducers, and another is the union of sperm and egg, , each of which is associated with specific differences in gene expression (=physiology, morphology, and behaviour) which we call the male and female sex respectively (Valenzuela 2008). An asexually reproducing female is twice as productive as a sexually reproducing female, since the sexual reproducer must spend energy on males for fertilization that do not themselves produce offspring (the ‘cost of males’; Maynard Smith 1971), so the benefits associated with sexual reproduction (such as genetic recombination) should be correspondingly large, and under strong selection (Lively and Morran 2014; Bachtrog *et al.* 2014; Valenzuela 2008). However, there is wide variation throughout the animal kingdom in the type and number of sex factors, which are the genetic and environmental bases that determine sex (Bachtrog *et al.* 2014; Valenzuela 2008). Given that animal life has a common origin, and that fundamental developmental processes (such as those involved in sex determination) should be subject to strong selection and therefore be conserved, this extensive variability in sex factors and determining systems is surprising (Beukeboom and Perrin, 2014; Uller *et al.*, 2007; Werren and Beukeboom, 1998).

Bull (1983) presents the classical genetic *vs.* environmental sex determination framework (Fig.1). In systems with environmental sex determination (ESD), sex is determined in the embryo largely in response to an environmental stimulus; examples of ESD in nature include temperature dependent sex determination (TSD) in many reptiles, where temperature during egg development controls hatchling sex ratio (Janzen and Phillips 2006). In systems with genetic sex determination (GSD), sex is primarily determined by the presence or absence of specific genetic factors such as chromosomes; for example, chromosomal sex determination (CSD) is prevalent in birds and mammals (Bull 1983; Bachtrog *et al.* 2014). In polygenic sex determination (PSD), sex is a threshold trait, underlain by a continuously distributed ‘sex tendency’, and influenced by genetic and environmental factors: those individuals with an underlying tendency above a threshold value develop into one sex, and those below the threshold develop into the other (Alexander *et al.* 2015), so that phenotypic expression of sex reflects the sum of genetic, environmental, and stochastic effects (Bulmer and Bull 1982; Alexander *et al.* 2015; Perrin 2016). The quality of sex factors, genetic or environmental, distinguishes GSD from ESD, and polygenic sex determination is distinguished from chromosomal sex determination by the number and magnitude of sex factors: there are 1 or 2 sex factors with large effect in chromosomal sex determination, compared to 3+ sex factors with minor effect in polygenic sex determination (Fig. 1; Uller and Helanterä 2011; Bull 1983).

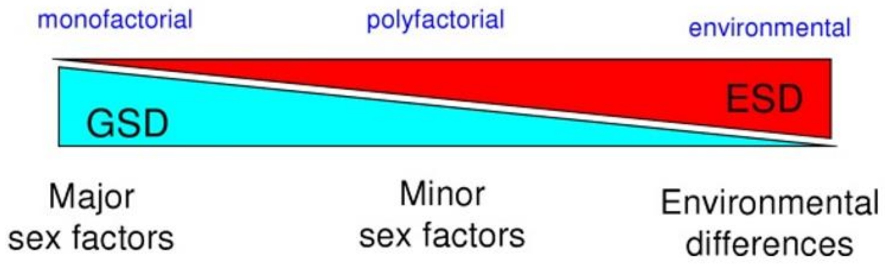

Figure 1: Schematic of type (genetic *vs.* environmental) and number (few to many) of sex factors in sexual reproducers: polyfactorial = polygenic sex determination, and monofactorial ~ chromosomal sex determination.

The proportion of male offspring at conception is defined to be the primary sex ratio; and variation in the primary sex ratio is closely associated with polygenic sex determination (Bull 1983). Historically, the way to distinguish between polygenic and environmental sex determination was by investigating the degree of sex ratio heritability as characterized by: wide within-family sex ratio variance, both maternal and paternal effects on sex ratio, and selectable sex ratios (Charnov and Bull 1977). More recently, quantitative trait loci mapping techniques have been useful in resolving the number and relative effect sizes of sex-associated loci (Alexander *et al.* 2015; Moore and Roberts 2013). Nonetheless, selection on sex ratio is an important part of polygenic sex determination (Bull 1983), and sex ratio variance can help identify cryptic polygenic systems. The study of sex ratio selection is governed by sex allocation theory (Bull 1983; West *et al*. 2002).

Sex Allocation Theory

The field of sex allocation research has been useful for modelling selection and adaptation, because it has a body of robust conceptual and mathematical theory and an informative trait, the sex ratio, which can be used to gauge how well reality matches theory (Fisher 1930; Charnov 1982; Grafen 2006; Trivers and Hare 1976; Trivers 1974; West *et al.* 2000). Sex ratio is tightly associated with fitness, and therefore selection (Seger and Stubblefield 2002; West *et al.* 2000). It is also relatively easy to sex and count organisms at both individual and population levels, so researchers can explore how sex ratio influences population demographics and life history trait evolution (*e.g.* Clark 1978; Trivers and Hare 1976).

The sex determining mechanism controls the sex ratio among zygotes (offspring), so the evolution of the sex determining mechanism depends fundamentally on the primary sex ratio; conversely selection on the sex ratio appears to be the dominating force in the evolution of sex determination mechanisms (Bull 1983). Sex ratios are measured at three key points in development (Fig. 2): conception/gamete fusion (primary sex ratio, PSR), hatching/birth (secondary sex ratio, SSR), and during adult life (adult sex ratio, ASR; Navara 2013).

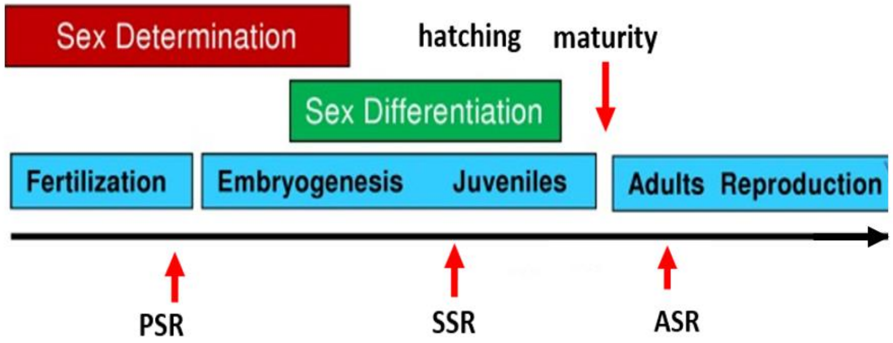

Figure 2: Schematic of ontogeny (black arrow) and sex determination. Primary, secondary, and adult sex ratio (PSR, SSR, and ASR respectively) are measured at corresponding stage (fertilization, hatching, and sexual maturity).

In his field-defining work Bull (1983) defined sex determination as “gender determination, the natural means by which a son or daughter is produced”. He conceptualized as a ‘trigger’ the initial sex determination event (compared to the complexity of the various molecular, genetic, and physiological processes that produce a male or a female from a zygote, which he simplified to ‘sex development’). This simplification allowed him to present an elegant, general perspective of sex determination mechanism evolution (Uller and Helantera 2011). However, in the case of polygenic and environmental sex determination this simplification can be problematic because sex may not be fully determined until after time has passed and the offspring are subjected to environmental effects that influence ‘sex development’. To address this, Uller and Helanterä (2011) define sex determination as “the processes within an embryo leading to the formation of differentiated gonads as either testes or ovaries”, which explicitly recognizes that the process of sex determination happens over time (*i.e.* includes sex development), and allows for the distinction that different selective processes can act on multiple levels (*e.g.* parent-zygote).

The evolution of sex determining factors is intimately connected to parent-offspring conflict, because parents and offspring are likely to have different ‘optimal’ sex ratios, and this intergenerational genomic conflict can affect the evolution of sex determination (Trivers 1974; Pen 2006).

At the population level, ‘optimal’ selection on sex ratio maintains species persistence (the evolutionarily stable strategy; Maynard Smith 1972). On average, every diploid, sexual species must transmit equal amounts of male and female genetic material to the next generation, so a sexual species should invest equally in male and female reproduction (Fisher 1930); if the amount of investment needed to produce male and female offspring of equivalent fitness is the same, this results in a 1:1 primary sex ratio. Deviations from 1:1 are selected against in a frequency-dependent way; this is known as Fisher’s Principle and it forms the basis for sex allocation theory. Sex allocation refers to the allocation of resources to male *vs.* female reproductive function (Bull 1983; Charnov 1982) including sex change (Charnov 1977) and the study of the sex ratio. At the individual level, selection favours parental ability to maximize their own fitness by altering parental investment towards either sex (Frank 1990), such as when the sexes require different amounts of parental investment to achieve equal fitness (*i.e.* when the 'costs' of the sexes differ). A classic example is when female ungulates produce more sons when they themselves are in good condition, because the reproductive output of large sons is higher than that of large daughters (Trivers and Willard 1973, Clutton-Brock *et al*. 1994). Therefore, at the individual level, the 'optimal' sex ratio maximizes parental fitness (Grafen 2006), possibly at the expense of its offspring and the larger population (Trivers and Hare 1976). This demonstrates the importance of perspective (parental *vs*. offspring) in sex allocation.

Sex allocation theory assumes parental control of sex determination (Bull 1983; West *et al.* 2002). Fisher's Principle predicts that parents allocate equal resources to the sexes during the period of parental investment (Fisher 1930); but if the sexes 'cost' differently, the parent can skew investment to maximize their own fitness rather than that of their offspring. But the parent is not the only one interested in the future sex of the offspring; if it is advantageous to the parent to produce the sex that maximizes their own fitness, then selection also favours opportunities for offspring to balance out the advantage gained by the parent. Unequal investment in the sexes can also set up conflict between siblings in the brood, since offspring always want to be the 'costlier' sex (that is, get more parental investment; Trivers and Hare 1976). This sexual conflict between siblings is expected to increase with an increasing degree of differential parental investment; the more a parent favours one sex, the greater the conflict between the sexes (Trivers 1974). The intensity of parent-offspring conflict is positively correlated with the period of parental investment (Trivers 1974), so the timing of an offspring's sex determination in relation to the period of parental investment is important when discussing sex allocation, especially in the context of environmental influence on sex.

The stronger the selection on the parent to skew investment (*i.e.* skew the sex ratio), the stronger the selection for offspring to counteract the parental effect (Trivers 1974). This selection for zygotic (offspring) control of sex determination is generally considered to be selection away from environmental effects on sex and towards genetic ones, specifically CSD: heterogamety is believed to resolve this parent-offspring conflict in favour of the offspring, because the coin-flip odds of sex determination by chromosome minimize potential for parental sex allocation strategies (Uller *et al.* 2007).

Opportunities for a zygote to influence the primary sex ratio are much more limited than the secondary or adult sex ratio, whereas the parent may have a great ability to influence the primary sex ratio until the end of the period of parental investment. However, if an offspring can delay sex determination until after the period of parental investment, it can experience the environment on its own terms, and the same environmental gradient that the parent was using to base its skewed sex allocation decisions could also be used by the offspring to influence its own sexual fate. A similar pattern is seen in the sex changers, where by changing sex as late in the juvenile stage as possible, individuals can become the sex that benefits most from the current environment (Bull 1983; Charnov and Bull 1989). A sex changer can maximize its lifetime reproductive output by timing sex change to coincide with the factor that affects its fitness (Vega-Frutis *et al.* 2014). In the same way, if offspring in a polygenic system can delay their own sex determination until after the period of parental investment, they can maximize their influence on their own sexual fate, relative to the influence of their parent. This would suggest that selection for zygotic control of sex determination was working away from CSD and towards ESD, which is contrary to most theory that I have been able to find on the subject (except Voordouw *et al.* 2002). To me, this represents an unexplained inconsistency in how sex allocation theory conceptualizes polygenic sex determination, and therefore makes the study of PSD extremely interesting.

The prevalence of chromosomal sex determination (and associated 1:1 primary sex ratio) in the animal and plant kingdoms, and theoretical models which predict that polygenic sex determination (and its associated sex ratio variance) should be transitory are seen as clear support for strong frequency dependent selection on sex ratio (Bull 1983; West *et al.* 2000). The frequency dependent nature of selection on sex ratio makes biased and variable sex ratios uncommon at the population level, except in special cases such as the haplodiploids (Uller *et al.* 2007; Kokko and Jennions 2008). Polygenic sex determination occurs rarely in nature, and so has been studied mostly theoretically in the context of conflict, as a transitional stage between GSD and ESD. Systems with polygenic sex determination include: European sea bass (Piferrer *et al.* 2005; Vandeputte *et al.* 2006), Lake Malawi cichlids (Ser *et al.* 2010; Parnell and Streelman 2013), East African cichlids (Roberts *et al.* 2016), lab but not wild stocks of zebrafish (Nagabhushana and Mishra 2016; Liew and Orbán 2014), the copepod *Tigriopus californicus* (Alexander *et al.* 2015), and possibly a chameleon (Ballen *et al.* 2016).

Polygenic Sex Determination: Stable or Transitory?

Transitions between sex determining mechanisms occur often (Bachtrog *et al*. 2014), and there are many examples of studies that focus on the relationship between parent-offspring conflict and transitions to and from GSD (*e.g.* Rigaud and Juchault 1993; Werren *et al.* 2002; Van Doorn and Kirkpatrick 2007; Kozielska *et al.* 2010; Kuijper and Pen 2010). However, there seem to be few studies that focus on how conflict in environmental sex determination systems can influence transitions between sex determination mechanisms (except Kuiper and Pen 2014).

This bias towards the study of genetic over environmental sex determination seems to persist for theoretical reasons (Uller and Helantera 2011). Theoretical modelling has predicted that selection should favour genetic sex determination over environmental sex determination in general (Rice 1986; Bull 1983), due to the high cost of stochastic fluctuations in sex ratio. The asymmetrical nature of geometric and harmonic means combined with large temporal or spatial variability in sex ratio is expected to reduce the adaptive advantage of matching offspring sex to environmental conditions over evolutionary time (Bulmer and Bull 1982), that is, to select against ESD (towards the left, in Fig. 1). Selection should also favour few factors with a major effect over many factors with minor effects (that is, CSD should be favoured over PSD) due to disruptive selection, which should allow genes with a large sex determination effect (such as SRY in mammals) to quickly invade a system with formerly strictly environmental sex factors (Bulmer and Bull 1982). This is also selection towards the left in Figure 1. What could select for ESD or PSD and away from CSD (that is, towards the right, in Fig. 1)? Selection on the sex ratio can cause and maintain environmental sex determination (Bull 1983; Janzen and Phillips 2006; Korpelainen 1990; Charnov and Bull 1977).

For a system to evolve toward environmental/polygenic sex determination (escape the chromosomal ‘trap’, and move towards the in Fig. 1), selection must favour sex ratio adjustment in response to particular environmental conditions as well as the evolution of a mechanism to allow for this sex ratio adjustment (that is, a sex determination mechanism or other sex ratio adjustment mechanism that can be influenced will evolve; Bull 1983). This selection for increased environmental influence on sex determination is strongest when the environment is patchy relative to male and female fitness due to local mate competition (LMC) or the patchy distribution of resources or predators (Charnov and Bull 1977; Hamilton 1967; Bull 1981). However, environmental sex determination is evolutionarily stable only if the correlation between fitness and the environmental variable of sex determination differs for males and females (Bulmer and Bull 1982). Furthermore, in the absence of selection for sensitivity to environmental input, regulation of sex determination is expected to evolve toward systems of a single locus with a major effect on sex determination, *i.e.* classic chromosomal sex determination (Bull 1983; Rice 1986), even when accounting for parent-offspring conflict over the sex ratio (reviewed in Werren and Beukeboom 1998; Uller *et al.* 2007; Uller and Helanterä 2011). So according to classical sex allocation theory, polygenic sex determination is supposed to be an unstable intermediate between ESD and CSD, unless there is some environmentally based (adaptive) selection influencing the sex ratio variance (*e.g.* Freedberg and Taylor 2007).

A more modern perspective on the evolution of sex determination mechanisms includes parent-offspring conflict at the level of the genome more explicitly. If a novel sex determining factor began to emerge (or if sex began to be associated with some new environmental factor, perhaps) *in the zygote alone* one might expect it to be selected against initially (because it would presumably interfere with ‘normal’ sexual development *e.g. via* antagonistic pleiotropy within the zygote’s genome; Uller *et al.* 2007). Therefore, one might expect that if a novel sex determining factor were to arise, it would need to be through some influence of the parent, resulting in genetic sex determination under both parental and zygotic influence. Such an example is the Seychelles warblers (Komdeur *et al.* 1997), which show evidence of maternal control over sex chromosome segregation. In this case, the novel sex determining factor is a product of the parent’s genome, not the zygote’s; the rise of novel sex factors may therefore experience different selective forces depending on this parent-offspring conflict. Nonetheless, even in systems with parent-offspring conflict over sex ratio, a relatively simple sex determination system under offspring control alone (like heterogamety; Werren *et al.* 2002) or under parental control alone (like haplodiploidy; Normark 2006) is likely to evolve (Uller *et al.* 2007). Systems in which the clutch sex ratio affects the female’s fitness or that of the clutch itself (that is, systems with parent-offspring conflict) tend to show increased zygotic influence on sex determination (Werren *et al.* 2002). The nature of parent-offspring conflict might dictate the conditions that are necessary for the evolution and maintenance of polygenic sex determination (Uller and Helantera (2011).

Chapter 2: Diet affects sex ratio in *Tigriopus californicus*

Introduction

***Tigriopus californicus*: A Model Organism**

One of the few species confirmed with polygenic sex determination is a copepod, *Tigriopus californicus*, a harpacticoid copepod 1-2mm long, which has ~6 loci on 5 different chromosomes that influence sex ratio (Alexander *et al.* 2014; Alexander *et al.* 2015). As expected in a polygenic system, *Tigriopus californicus* has extremely variable and generally male-biased within-family (individual level) sex ratios (Voordouw and Anholt 2002b; Voordouw *et al.* 2005; Voordouw *et al.* 2008). Population level sex ratios are also slightly male biased (*e.g.* 0.54; Egloff 1966) and variable in the wild, with sex ratios ranging from 0.07-0.84 with a left skew reported by Egloff (1966). Lab population sex ratio is also variable (*e.g.* <0.2 to >0.9; Voordouw *et al.* 2005). The heritability of sex-ratio-influencing loci explains about 0.19 of the possible 0.75 (a quarter of variance will always be attributed to the binomial nature of the sex ratio; Mittlbock and Schemper 1987). Consequently, about half the sex ratio variance in the *T. californicus* system is not due to the known genetic and stochastic factors, but presumably must be due to environmental effects.

Temperature, photoperiod, nutrition, density, humidity, pH, UV light, and parasites are some examples of environmental factors that can influence sex determination in invertebrates (Korpelainen 1990). In copepods generally, association studies show that temperature and resource availability are the two environmental factors that best account for sex ratios (Gusmão *et al.* 2013). In *Tigriopus californicus*, about a third of *T. californicus* lineages show slight sex ratio sensitivity to temperature (a 5% increase in the proportion male from 15-22 °C; Voordouw and Anholt 2002a). This hint of environmental sex determination, combined with a weak pattern of sex ratio oscillation in the wild (Anholt, unpub.) suggests a seasonal component to some of the sex ratio variability in this system (Bateman and Anholt 2017). Salinity (Egloff 1966), UV-B irradiation (Chalker-Scott 1995), and pressure (Vaquier and Belser 1965), have also been reported to influence sex ratio, but none of these results have been replicated to my knowledge.

From an ecological perspective, *Tigriopus californicus* inhabit the patchy and ephemeral habitat that are the supratidal pools on rocky outcrops from Baja California to Alaska (Burton 1985), often at high densities: each pool within an outcrop can be viewed as a habitat patch, with dispersal and colonization success being influenced by patch environmental characteristics (Altermatt *et al.* 2012). For example, pools vary in temperature, salinity, oxygen level, and species composition (Egloff 1966; Dybdahl 1995; Lee and Taga 1988); population extinction occurs when pools dry up, whereas colonization happens toward the waterline when pools flood (Vittor 1971), and away from the waterline by hitchhiking on shore crabs (Egloff 1967, Dybdahl 1994). Pools on a single outcrop represent a metapopulation that differs genetically from populations on a separate but adjacent outcrop (Burton and Feldman 1981). The transcriptome of *T. californicus* has been published; many genes are under positive selection (Barreto *et al.* 2011), suggesting that metapopulations can adapt to environmental variables that differ between outcrops; for example, Kelly *et al.* (2013) and Willett (2010) showed local adaptation in the copepod's ability to respond to thermal stress. This population structure reflects the importance of patchiness of the habitat, which is assumed for much of sex allocation theory (Hamilton 1967; Charnov 1982).

Tigriopus californicus breed year-round, producing a dozen or more ovisacs of about 20 to more than 100 eggs each over their lifespan of 2-3 months: females fertilize eggs using sperm stored from a single mating, which happens at their terminal moult by the male who has been guarding them as they matured (assuring single paternity of all offspring from a given female; Burton 1985). Females cannot choose to stop producing eggs once mature, which combined with year-round breeding has been suggested to result from strong selection based on the ephemeral nature of their habitat (Vittor 1971).

They have 6 naupliar stages and 6 copepodite stages with the final stage being the adult (Hicks and Coull 1983). Generation time is about 3-4 weeks in the lab (Burton 1985).

There are several challenges working with *Tigriopus californicus*. Both males and females are diploid and have no visible sex chromosomes (Ar-Rushdi 1963) and because of this, they cannot be sexed until they are fully mature and sexually dimorphic, necessitating either the assumption that the adult sex ratio is identical to the primary sex ratio, or the ability to account for the sex-differential larval mortality which might cause them to differ. Secondly, we do not know exactly when sex is ‘determined’ either absolutely (though it is certainly before the C1 stage) or relative to the period of parental investment (which ends upon hatching, and before offspring are fully committed to either testis or ovary development). Thirdly, it is unknown to what degree sex determination is under parental *vs.* zygotic control (Alexander *et al.* 2015); that is, the degree of parent-offspring conflict in the system. Finally, it is currently unknown whether or to what degree female *Tigriopus* can allocate sex, and how female influence on sex ratio might interact with life history traits (such as fecundity and survival).

According to Alexander *et al.* (2015), steps should be taken to determine where the rest of the sex ratio variability in *Tigriopus californicus* is coming from, if not from genetic sources then presumably from environmental ones.

Resource Allocation in *Tigriopus*

Sex allocation theory can be viewed under the larger umbrella of life history theory, which is the study of how an animal should allocate resources to reproduction to maximize its own fitness over its lifetime (Roff 1992; Stearns 2000). In iteroparous species (that breed more than once before death) there is a trade-off between an individual’s current fecundity and future survival, since reproductive effort placed into the current breeding episode decreases effort for future reproduction and survival (Taylor 1991; Stearns 1992). Survivorship is generally inversely proportional to previous reproductive effort regardless of sex (Murdoch 1966).

A useful way to think of the physiological trade-off between growth/survival and reproduction is capital *vs*. income breeding (*e.g.* Houston *et al.* 2007). Capital breeding describes the situation in which reproductive effort is ‘financed’ using stored capital; in an invertebrate like *Tigriopus*, this could describe the strategy of storing specific nutrients and/or energy for reproduction while still immature, and once mature, spending this reserve consistently during the lifespan (*i.e.* a linear or curvilinear pattern of energy expenditure). In income breeding, reproductive effort is ‘financed’ using energy gained concurrently (as reviewed by Stephens *et al.* 2009). In *Tigriopus*, this could describe the strategy of focussing on growth as a juvenile rather than on storing energy for later; as adults then, they must have constant access to energy (and/or specific nutrient) sources if they are to reproduce. This pattern of energy expenditure might therefore show up as fluctuating sex ratios/clutch sizes, or sex ratios/clutch sizes dependent on resources (diet).

Which of these two strategies best approximates resource/sex allocation of *Tigriopus californicus* females is unknown. However, because of the ephemeral nature of *Tigriopus* habitat, an abundance of resources now could be capitalized upon by females with an income breeding strategy, compared to those with a capital breeding strategy. So female *Tigriopus* can potentially choose whether to have large clutches and lots of females early in life, but die sooner than if they had smaller clutches and more males early in life, and this choice is limited by the physiological ‘capital’ or ‘income’ breeding styles that underlie metabolism. Age itself may influence the female’s accuracy at carrying out her sex allocation decisions: *Nasonia* is a haplodiploid parasitic wasp, and she gets less accurate at physically controlling fertilization (which determines sex ratio) with age (Ueno 2014).

The period of parental investment determines the temporal window in which parents can influence their own fitness by adjusting the offspring sex ratio (Fisher 1930; Seger and Stubblefield 2002). For the male *Tigriopus*, his investment is solely in mate-guarding and subsequent fertilization upon the female’s final moult (Burton 1985) so it is difficult to see how a male could influence sex ratio, except through his sperm (which gets stored and used to fertilize all clutches for the duration of the reproductive lifespan of the female; Burton 1985). There is no evidence of sex ratio distorters at work (based on the lack of sex ratio response to treatment with antibiotics; Voordouw *et al.* 2008).

For the female, opportunities to allocate sex are more obvious: a) during egg provisioning (vitellogenesis) inside the ovaries, b) fertilization and oviposition, and c) ripening in the ovisac attached to the female’s abdomen. Sex ratio and fecundity (or clutch size) are closely linked (Moreno-Rueda *et al.* 2016).

Resources and Egg Provisioning in Copepods

Egg size is a widely used predictor of reproductive investment in animals (Winkler and Wallin 1987; Sinervo and Licht 1991; Bernardo 1996). By allocating more resources to offspring of one sex (males or females in larger eggs, for example) parents can alter selection acting on zygotic sex determiners, such as in kestrels (Anderson *et al.* 1997) and spotless starlings (Cordero *et al.* 2001). In many bird species, egg size declines along the laying sequence, probably because of maternal resource limitation (Slagsvold *et al.* 1984). Unlike in birds, insects, and other crustaceans however, the details of egg provisioning in copepods and the hormones and associated mechanisms that regulate gametogenesis, oocyte development, egg production and larval development are largely unknown (Poulet *et al.* 2007).

Nonetheless, it is known that vitellogenesis and subsequent oocyte maturation are highly energy-demanding and especially affected by nutrient supply: in calanoid copepods, for example, there are two stages of vitellogenesis and both are linked to maternal diet (Poulet *et al.* 2007). In the harpacticoid copepod *Euterpina acutifrons*, which has a similar reproductive strategy to *Tigriopus californicus*, daily egg production was about 32% of the biomass of the female (Zurlini *et al.* 1978). *Tigriopus* females produce eggs whether they are fertilized or not (though it is unlikely they are ever not fertilized in the wild (Burton 1985), so the total number of clutches produced in their lifetime is likely not under their individual control.

If this is the case and if chicks are also sexually dimorphic, mothers may increase fitness by allocating the largest sex to larger first laid eggs (*e.g.* (Alonso-Alvarez 2006). In the copepod *Cyclops kolensis*, old females produce smaller eggs than young females, possibly because of a reduction in their lipid reserves or other products (Jamieson and Santer 2003); investment in embryos therefore decreases as females age, and if the sexes 'cost' differently, this maternal aging might be associated with a change in sex ratio as well (Burris and Dam 2015). While some copepods are known to increase the size of their embryos at the expense of the number of embryos produced (Guisande *et al.* 1996), *Tigriopus* egg size seems to be canalized: food scarcity reduced individual clutch size, and food abundance increased lifetime fecundity and individual clutch size (Vittor 1971). The importance of resource quantity and quality is expected to be most closely associated with clutch size, rather than egg size or total number of clutches, in *T. californicus*.

Aside from egg size differences, specific products placed in the egg by the female can also be important during early sex development since the zygotic genotype is not expressed during early mitotic division (Werren and Hatcher 2000). Maternal provisions are presumably very important in *Tigriopus* early naupliar stages, since active feeding is not observed prior to the N-III stage (Lewis *et al.* 1997), and nauplii can live up to 4 days before starving (Lee and Taga 1988), suggesting that any maternal gene products (proteins, lipids, or mRNA) placed in the developing egg could have significant effects on sex determination (Werren and Beukeboom 1998). In *Tigriopus fulvus*, females and eggs differ in fatty acids composition (Carli *et al.* 1984), and fatty acid profiles of copepods tend to closely track their food supply (Evjemo *et al.* 2008), indicating that the females must be actively allocating particular assemblages of fatty acids to eggs. In *Euterpina acutifrons* (another harpacticoid copepod), amino acids also differ between females and eggs and the environmental nutrient supply (Guisande *et al.* 2000). Clearly, female copepods can control egg provisioning to some degree, and if these nutrients were sex-differentially associated with fitness, this could represent an avenue for the female to adjust sex ratio in her own favour.

Aside from during egg provisioning, a female could influence sex ratio during fertilization and oviposition. *Tigriopus japonicus* eggs are fertilized as they exit the genital pore (Takano 1971), which connects the ovary to the external membranous ovisac via an umbilical style cord (Kahan *et al.* 1988). There is no evidence that suggests fertilization is associated with sex ratio differences in any *Tigriopus* species (as occurs in haplodiploidy).

Finally, a female could influence sex ratio of her clutch while it is attached to her abdomen as it ripens. Maternal influence on their clutches prior to hatching through hormone diffusion has been demonstrated in *Tigriopus japonicus;* females inhibit hatching of their clutches at high population densities, and this is presumably accomplished hormonally through the umbilicus (Kahan *et al.* 1988). This inhibition of hatching seems to occur in *T. californicus* as well (pers. obs.).

Hormones of maternal origin may prevent hatching, but they also may stimulate ripening (and hatching comes only after ripening). The hormones involved in sex development and egg ripening are unknown in *Tigriopus californicus*, but under normal development, the female oviposits a dark green mass of eggs, which turns orange-red and translucent is it ripens and the pigment astaxanthin is freed from protein bonds (Ambati *et al.* 2014; Cianci *et al.* 2002; Lewis *et al.* 1997). The ripening process takes 2-5 days at ~20°C (Burton 1985), and at some point, eggs become ripe enough to continue ripening on their own if they are removed from the female, breaking the umbilical link (this is necessary because in the lab, females will eat their own offspring if they are not separated, which can interfere with sex ratio measurement).

I made three observations which suggest that females stimulate ripening hormonally: (1) Females will sometimes drop green egg sacs which never ripen (these are often assumed to be unfertilized), and if an egg sac is removed from a female too early during ripening, it will never ripen or hatch. (2) When females died and remained attached to their green clutches, clutches ripened and hatched, as though the female was alive. (3) However, if the clutch was disconnected from the dead female, but left adjacent, the green clutch remained unripe. This was true when the female died naturally (initial observation) and when I killed the female with pressure to the head (taking care not to allow puncture which would release chemical signals and confound the trial). These three observations suggest to me that the female is providing the eggs with something which stimulates ripening, that is passively diffused (since it was effective whether the female was dead or alive) through the umbilicus connecting her to her clutch (since an adjacent dead female was not sufficient to ripen green eggs). A hormone would be a perfect candidate.

Sex Allocation Hypotheses in *T. californicus*

The study of sex allocation has been fruitful (see reviews by West *et al*. 2000, and by Rosenfeld and Roberts 2004), without needing to regard the details of the sex determination mechanism, so I initially approached the *Tigriopus californicus* system to evaluate the support for three possible sex allocation situations that might be occurring: condition dependence (*e.g*. Trivers and Willard 1973), local resource competition (Clark 1978), and social mediation (e.g. Cole and Shapiro 1995).

Trivers and Willard (1973) proposed that maternal condition could influence sex ratio if 1) offspring condition is correlated to maternal condition 2) differences in condition endure into adulthood and 3) adults of one sex benefit more than adults of the other sex from being in better condition (*e.g.* red deer [Clutton-Brock *et al*. 1994]; possums [Isaac *et al*. 2005]). This third condition is a different way of saying the 'costs' of the sexes differ, since it takes proportionally more investment from the parent to make one sex as fit as the other (West *et al.* 2002). A modification of this maternal condition-dependence relates sex ratio to the condition of the environment directly (although this effect may still be mediated by the female). For example, in the Seychelles warbler (*Acrocephalus seychellensis*), females breeding in high quality territories produce more daughters, whereas those breeding in low quality territories produce more sons (Komdeur *et al.* 1997). Similarly, tawny owls lay female biased sex ratios in habitat likely to have a lot of voles (Appleby *et al.* 1997).

A key assumption of condition dependence is that the costs of the sexes differ. It turns out that assuming females are more expensive than males (or one sex is more expensive than the other) based on size is a widespread practice in sex allocation literature (*e.g.* Moreno-Rueda *et al.* 2016; Becheikh *et al.* 1998; Peterson and Roitberg 2006; Santolamazza-Carbone *et al.* 2007; Bradbury and Blakey 1998) and few researchers have offered detailed justification for their assumption. The differential equilibrium hypothesis explains intersexual variation in body size as the result of the balance between selection for larger size (*i.e.* fecundity selection in females and sexual selection in males) and viability selection for smaller size (Blanckenhorn 2000; Berner and Blanckenhorn 2007; Romero *et al.* 2014). While viability selection restrains body size in both sexes, *Tigriopus* males can potentially choose whether to mature earlier and guard females sooner, or mature later, and hope that their larger body size is enough to oust the smaller early-maturers. For females, however, the importance of female body size for egg production in copepods is well established from taxonomic surveys, and increasing egg production is the best way for a female to improve her fitness (Kiorboe and Sabatini 1995; Hopcroft and Roff 1998), suggesting that for female copepods, it is always better to be bigger (within the constraints of viability selection). So for male copepods, the trade-off between body size and age at maturity seems to afford more choices than for female copepods

(Stearns and Koella 1986). In parasitic wasps female offspring are more expensive because they gain a greater fitness benefit from extra resources and larger body size (West *et al.* 2000; West and Sheldon 2002; Santolamazza-Carbone *et al.* 2007). Female *T. californicus* are larger than males (Lewis *et al.* 1997) as in most harpacticoid copepods (Hicks and Coull 1983). The high cost of constant vitellogenesis in combination with their larger body size suggest that *T. californicus* females are 'more expensive' than males.

Another sex allocation strategy that may be occurring in the *Tigriopus* system is local resource competition (LRC), wherein a female produces more of the sex that disperses as she ages, because she should minimize competition between herself and her own philopatric offspring (Clark 1978), so this pattern would be reflected by a correlation between age and sex ratio. This strategy does not depend on a cost difference between the sexes, but rather on which is the dispersing sex: according to Dybdahl (1994), successful colonists in *Tigriopus* pools are about three quarters female, suggesting that females are the dispersing sex; therefore, an increasingly female-biased sex ratio is expected as females age if LRC is at work in the *Tigriopus* system.

The Trivers and Willard-style condition dependence hypothesis is often applied to sex ratios at the individual level, whereas LRC is often applied at the population level (Ward, 2003). In reality, both condition dependence and LRC processes are happening together; for example, LRC seems to be at play along with condition dependence in the Seychelles warbler system, since daughters usually remain as helpers and enhance the future reproductive success of their parents, but sons disperse and do not decrease the future fitness of their parents by competing with them for resources (Komdeur *et al.* 1997). Note that in both cases (condition dependence and LRC), there is an implied reliance on the resources available to females: in the former, the better condition of the mother depends on her access to resources, and in the latter, the competition between mother and offspring is exacerbated or ameliorated by availability of resources (*i.e.* the condition/quality of the environment).

Another such cue upon which a female might make a sex allocation decision is the availability or proportion of possible mates/conspecifics, referred to as social mediation of sex allocation. Social mediation is seen in hermaphroditic reef fishes; local sex ratio influences when sex change occurs (*e.g.* Cole and Shapiro 1995; Liu and Sadovy 2004). In dioecious species, this relates to the concept of local mate competition, which states that in populations with patchy habitats, a female can increase her lifetime fitness by producing offspring of the rarer sex, provided that sex can then disperse and find a surplus of mates in another patch (Hamilton 1967). To date, there have been 4 separate investigations of social mediation in *T. californicus* that I am aware of, including chemical and touch-based sensory modalities, (Anholt lab: Marie Vance, Travis Tai, Erin Hornell, Megan Ljubotina) and only one (Tai 2014) found a slight effect of local sex ratio on resultant female clutch sex ratios, where females produced sex ratios more biased toward the rarer sex as predicted. Social mediation is often associated with cost differences between the sexes (as in the parasitic wasps; Charnov *et al.* 1981), which reflects sex differential environmental selection. I therefore expected that environmental quality might be more closely associated with sex ratio variance than local sex ratio, so I focused my study on the role of resources in *Tigriopus* reproduction.

It is necessary to record sex ratios of subsequent clutches of a given female (a sex sequence) to learn about individual level resource and sex allocation decisions. A recent paper by Ambrosini *et al*. (2014) shows how tracking sex ratios over multiple clutches produced by individual females (= sex sequences) could give insight into broad maternal sex allocation patterns. This sex sequence analysis can compare a set of three alternative hypotheses to a null (no change of sex ratio over the sex sequence): (1) the clutch sex ratio changes linearly along the sex sequence, depending on clutch laying order alone (as with sperm age; Olsson *et al.* 2007) or maternal age (Ross *et al.* 2011); (2) the clutch sex ratio changes according to the sex ratio of the previous clutch alone, and not on laying order (as might occur in social mediation or in condition dependent sex allocation, where patch characteristics are expected to be more important than age); (3) the clutch sex ratio changes linearly along the sex sequence and according to the sex ratio of the previous clutch (if more than one of these factors influences sex ratio; Ambrosini *et al.* 2014). This sex sequence analysis could provide some information on the background income *vs*. capital breeding style of *T. californicus*: if the former, I expect clutch sex ratio to change linearly, and therefore depend on its position in the laying order, whereas if the latter, I expect clutch to be dependent solely on the previous clutch's sex ratio (consistent with a role for nutrient depletion and restocking independently of laying order).

Tigriopus californicus can be raised for many 14 generations on flaked TetraMin fishfood in the Anholt lab: other labs have fed *Tigriopus* yeast, mulberry leaves, rat food, dried shrimp, *Actinobacter* bacteria, *Tetraselmis*, *Isochrysis*, *Rhodomonas*, *Platymonas* (=*Thalassosira*), diatoms etc. (list in Table II of Lewis *et al.* 1997). One gut content analysis of wild *Tigriopus californicus* revealed (in order of abundance and not further specified): (1) diatoms, (2) green and blue-green algae, (3) filamentous green algae (Egloff 1966): a second gut census included (1) the green alga *Chlorococcum*; (2) the cyanobacterium *Oscillatoria*; (3) the dinophyte *Oxyrrhis*; and (4) *Euplotes* protozoans (Huizinga 1971). Based on gut contents, *T. californicus* phytoplankton prey species are clearly variable and likely vary by patch, fulfilling the requirement for patchy distribution of resources upon which much of sex allocation study is based (*e.g.* Hamilton 1967; Charnov *et al*. 1981).

The object of this thesis is to compare potential effects of female *vs*. offspring on the offspring sex ratio over the course of a sex sequence, in relation to diet. In the first experiment, the Algae experiment, I used two diets, one a 'high' quality live algae, the other 'low' quality flaked fish food, and I recorded sex ratio of up to 8 clutches (limited by time and resources, not mortality). Note that I use the terms 'high/low quality' as a simplification: TetraMin fishfood is not poor quality food, and I have it designated 'low quality' because it is further from what these copepods eat in the wild, whereas the live algae is closer to their natural diet, which is designated 'high' quality.

Diet-independent effects on sex ratio could include aging itself, which is associated with general decline in condition (senescence) and predicts a male-biased sex ratio as the female ages (Fig. 3, top), as well as LRC, which predicts a female-biased sex ratio with age since females are the dispersing sex (Dybdahl 1994; Fig. 3, top). Sex ratios dependent on clutch diet, with no maternal effect at all, should show no change in sex ratio with parity, since the information of position in the laying order could only originate with the female (Fig. 3, middle). Sex ratios dependent on maternal diet might resemble Trivers and Willard-style condition dependence: *Tigriopus* females may produce sex ratios biased towards the more 'expensive' sex (*i.e.* females) as their condition improves on the high quality diet (Fig. 3, bottom). I also expected clutch sizes to be larger when females ate the high quality diet and were in better condition.

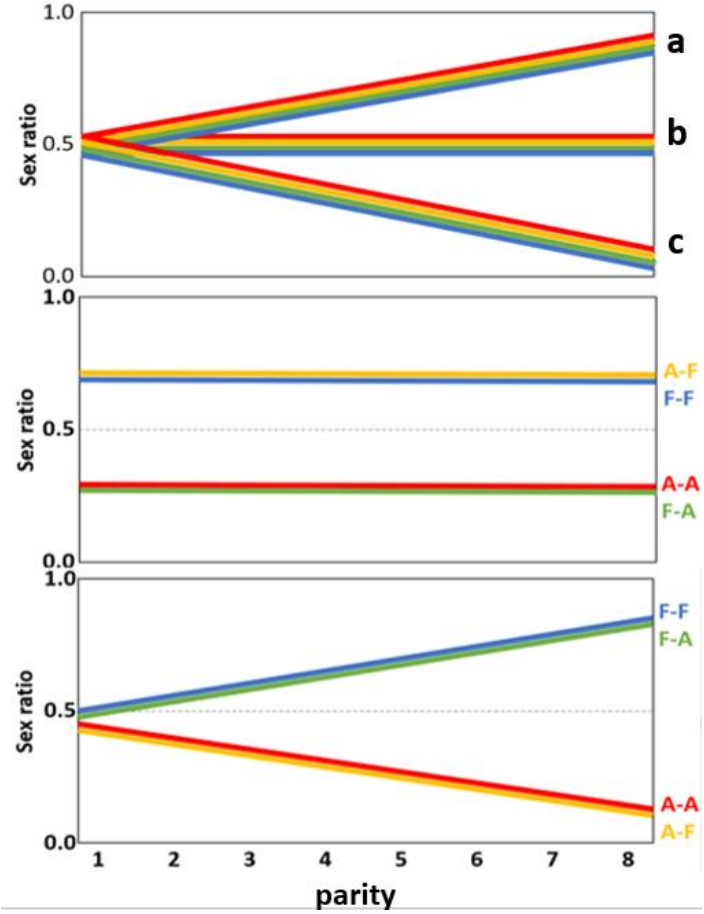

Figure 3: A schematic of some possible hypotheses for the relationship between sex ratio and parity (or maternal age). TOP: Diet-independent pattern of sex ratio change with clutch number (parity), where (a) maternal age decreases her condition (b) the null, no sex ratio change (c) decreasing sex ratio = increasing disperser sex = evidence of LRC. MIDDLE: Sex ratio is dependent on clutch diet, with no maternal effect. BOTTOM: Sex ratio increases/decreases according to maternal diet (as proxy for her condition, evidence of Trivers and Willard-style condition dependence). A-A, A-F, F-A, and F-F and the corresponding coloured lines represent four possible combinations of female diet-clutch diet, where A= live algae diet, and F= flaked fishfood diet. Dashed line represents 1:1 sex ratio, below which is female-biased, and above, male-biased.

Methods

In early April 2015 I collected wild *Tigriopus californicus* from Cattle Point, Victoria, BC, Canada (48° 26' 13.1748" N 123° 17' 36.9816" W). Copepods acclimated for about 2 weeks in their natal tidepool water to lab conditions. I indiscriminately selected 216 gravid females with ripe ovisacs from the population and separated them from their ovisac, with each (female and ovisac/clutch) being assigned to one of two food treatments: 108 females were assigned each to the algae treatment (A – live *Isochrysis galbana* Parke) or the fishfood treatment (F – TetraMin™ fish food mixture), and their ovisacs (=clutches, once removed from female) were split into two groups of 54 (multiplied by up to 8 clutches per each of these 54 females) and assigned to either algae or fishfood diet treatments in a full factorial design. I observed each of the 216 females for the next month, removing ovisacs as they ripened, and assigning them to their respective food treatments; in this way, I removed a maximum of 8 ovisacs from each female (limited by time and resources). Clutches were raised to maturity and the number of males, females, and juveniles per clutch was recorded. From this, the clutch sex ratio (=proportion male = #males / [#males + #females]) and clutch size (= #males + #females + #juveniles) were determined when most of the clutch was mature. Females were kept individually in 60mL wells, and clutches were raised with all siblings together in a 60mL well (so that density of nauplii, *i.e.* clutch size, was variable); females and clutches both were kept at ambient temperature in the lab.

Diet Regimes

The females and clutches in the fishfood treatment received the TetraMin mixture often used in *Tigriopus* studies (*e.g.* Alexander *et al.* 2014): I made a slurry of 0.2g TetraMin™ Tropical Flakes, 0.2g Omega One™ Super Veggie Kelp Flakes, and 40mL filtered salt water (filter 0.5µm); newly hatched nauplii were fed 30µL, and females 50µL, as needed, which was approximately every 3 days, or when the culture wells were visibly empty. The females and clutches in the algae treatment received live, hand-raised *Isochrysis galbana* Parke (inoculate obtained from NCMA, CCMP1323) in filtered salt water with Provosoli’s algal culture medium; newly hatched nauplii were fed 500µL, and females 1000µL as needed (approximately every 2-4 days). All algae were taken from 500 mL monospecific batch cultures in log-phase growth. To control for differences in micronutrients between the algae culture medium and the fishfood slurry, I added the same amount (500µL or 1000µL) of fresh algal culture medium (with no algae) to the fishfood treatments. Late in the experiment (when the 6-8th clutches were just reaching copepodite stages), it was necessary to feed the algae treatment *Pavlova lutheri* instead of *I. galbana* though this unlikely to have influenced my resultant sex ratios since sex is determined already by the copepodite stage (Egami 1951). I fed all diet treatments to satiation, to avoid confounding energy limitation with resource limitation.

Penstrep™ (=5000 units penicillin and 5000 µg streptomycin/100mL: I used 5mL per 1L sea water) was added to clutches in all diet treatments, because early naupliar stages are susceptible to mortality from bacterial or fungal growth (*e.g.* Alexander *et al.* 2014).

Sexing and Larval Mortality

I sexed clutches when females bearing ovisacs became visible, indicating that clutches were mostly mature. Males were easily identified by their specialized knobby grasping antennae, and females by their larger size and long, slender antennae. Juveniles, C4-5 copepodites, were distinguished from females by their smaller size and shorter antennae. I counted all adult copepods as male or female, whether dead or alive, but not dead or living copepodites, because they cannot be sexed (after Voordouw *et al.* 2005). Data were compared with and without the larval mortality correction (where juveniles are assigned the rarer sex and included in analysis, *vs.* not being included at all; see Voordouw and Anholt 2002a) and were very similar, so uncorrected data were used for analysis and are presented. Clutch 1 mortality was less than 1%, and otherwise remained low for the duration of the experiment.

Temperature

All diet treatments were kept at ambient temperature in the lab for the duration of the experiment, interspersed in blocks, such that they all experienced the same temperature regime. Temperature was logged using iButtons (Maxim Integrated, DS1921G Thermochron) to ascertain that lab temperatures remained relatively constant. Mean daily temperature in the lab ranged from 18.4 to 21.4 degrees C, while fluctuations during the day caused temperatures by the hour ranging from about 17-24 degrees C, well within the normal range for these copepods.

Statistical Analyses

I used two different analyses, both GLMMs, to investigate the factors contributing to sex ratio variance. GLMMs can account for differing clutch sizes and missing data, they can model binomial error structure (=variance), which suits proportion data such as sex ratio and they allowed me to use a random effect on the intercept (female ID) to account for the non-independence of data from the same individual (Bolker *et al.* 2009; Zuur *et al.* 2009). This should increase my ability to distinguish sex ratio variation due to my experimental manipulation (that is, female diet, clutch diet, and parity, or clutch laying order; Table A) compared to the effects of say, family lineage, in what I will call the Diet analysis. Clutch 1 sex ratios were not included in this analysis, because clutch 1 sex ratios reflect the unknown diet of the female rather than the experimental diets, and female diet is modelled as a fixed effect in this GLMM. I will call the second analysis, also a GLMM model comparison, the Sex Sequence analysis, which was carried out on the full data set including clutch 1 (since female diet is not a fixed effect in this analysis). Whereas the Diet analysis should inform me about the effect of female and clutch diet, the Sex Sequence analysis can be best interpreted as a breakdown of the pattern of sex ratio dependence within a sex sequence (*i.e.* over the laying order, with parity), outside of the context of diet. I expect this analysis to be informative regarding *e.g.* age-based effects on sex ratio compared to nutrient depletion and restocking.

To estimate the explanatory value of the best model compared to the null (the goodness of fit) I used the formula:

$$\textbf{pseudo-}\mathbf{R^2 = 1 - (\textbf{log likelihood[best model] / log likelihood[null model]})}$$

which yields a value between 0 and 1 and can be interpreted as similar to a Pearson’s R^2 value. Another gauge of this is to compare the residual deviance of the best model against the residual degrees of freedom, with the expectation that the two values should be similar in situations with good model fit (*e.g.* Voordouw and Anholt papers).

I also used ANOVA to investigate a secondary fitness-related response variable, total clutch size (males + females + juveniles), which reflects a) initial clutch size, dependent on female fecundity and b) juvenile survival, the corollary of larval mortality.

Results

I collected wild female *Tigriopus californicus* and fed them either live algae or fishfood; I removed up to 8 of their clutches and raised them to maturity eating either algae or fishfood, in a factorial design. I sexed them once mature, and recorded clutch sex ratio and clutch size (incorporating female fecundity + larval survival). I conducted two GLMMs, one on data from clutches 2-8 (the Diet analysis), the other on the full dataset including clutch 1 (the Sex Sequence analysis). I used ANOVA with clutch size (fecundity -- larval mortality) to confirm the condition of females, expecting larger clutch sizes from females who ate algae.

The Diet Analysis

I recorded the clutch sex ratio (of clutches 2-8) as the response variable, and, using AIC, chose the best GLMM from a nested candidate model set (Table 1) which included three predictor variables (female diet, clutch diet, and parity) and a random effect (female ID).

Table 1: Candidate model set list and AIC values fromthe Diet analysis (GLMMs with binomial error, fixed effects 'maternal diet', 'clutch diet', and 'parity' (=laying order).

Model Structure	DF	AIC	ΔAIC
1. clutch diet * parity * female diet	9	8989.9	0
2. clutch diet * parity + female diet * clutch diet	7	9009.5	19.6
3. clutch diet * parity + female diet	6	9121.9	132.0
4. clutch diet * parity	5	9124.4	134.5
5. clutch diet * female diet + female diet * parity	7	9752.3	762.4
6. clutch diet * female diet + parity	6	9831.3	841.4
7. clutch diet * female diet	5	9908.4	918.5
8. female diet * parity + clutch diet	6	9933.1	943.2
9. female diet * parity	5	9936.9	947.0
10. clutch diet + female diet + parity	5	10043.1	1053.0
11. clutch diet + parity	4	10045.5	1055.6

12. female diet + parity	4	10054.0	1064.0
13. parity	3	10057.4	1067.4
14. clutch diet + female diet	4	10102.1	1112.2
15. clutch diet	3	10103.5	1113.6
16. female diet	3	10107.2	1117.3
17. null	2	10109.2	1119.3

According to the terms of the best model, sex ratio changed with parity (=laying order) for all four treatment combinations. Clutch diet determined the direction of sex ratio change: clutches that ate fish food became more male-biased with parity, whereas those that ate algae became more female biased; Table 1, Fig. 4). There was a clear effect of maternal diet on sex ratio as well, evidenced most clearly by the fact that sex ratio changed at all (since clutches have no way to know their position in the laying order except through their mother). The influence of maternal diet can also be seen in Fig. 4: when clutches ate fish food, the ones whose mothers ate algae had sex ratios about 0.10 lower than those whose mothers ate fish food (for all clutches 2-8). However, when clutches ate algae, the ones whose mothers ate fish food had the lower sex ratios by about 0.03 than the ones whose mothers also ate algae. This suggests that the maternal diet had a greater influence on sex ratio when clutches ate the fish food (worse quality) diet than when clutches had access to the good quality algae diet themselves.

This model had a pseudo-R^2 value of 0.112, indicating that my explanatory variables together with the random effect explain about 11% of the total variation in sex ratio, slightly less than the 19% that is accounted for by purely genetic sex ratio heritability (Alexander *et al.* 2015). (These values are not derived from the same data or statistical methods and are not strictly comparable, but still suggest that the resource-based environmental influence on sex is almost as strong as the genetic influence on sex). I was unable to find more comparable descriptions of variance using GLMs or GLMMs in the literature; other papers (including those from the Anholt lab) report p-values to give readers a joint frequentist/model comparison approach to statistics. My approach is strictly model comparison, and I can’t compare p-values to my pseudo-R^2.

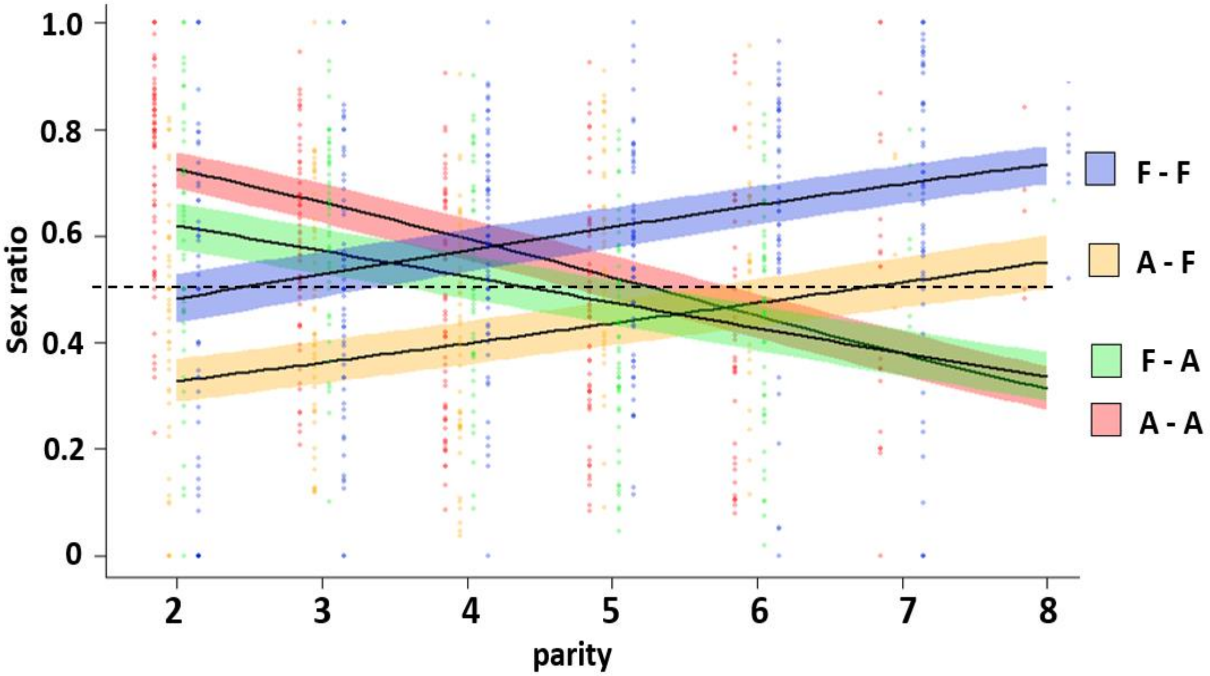

Figure 4: Mixed effects model of *T. californicus* sex ratios, when females and clutches were fed either high quality live algae or 'low' quality fish food in a factorialized design (n=54 per treatment combination). Legend identifies diet treatment combinations as 'female diet-clutch diet' with A= algae and F= fish food. Model formula: sex ratio ~ clutch diet*parity*female diet, with female ID as the random effect, and a binomial error structure. Points represent observed sex ratios, whereas black lines and coloured polygons represent mean sex ratio predictions and confidence intervals respectively (based on the model's posthoc best linear unbiased predictors). Dashed line represents 1:1 sex ratio, below which is female-biased, and above, male-biased.

I ran ANOVA to evaluate the difference in mean clutch size between the diet treatments (Table 2, Fig. 5).

Table 2: Analysis of variance of clutch size in relation to maternal and clutch diet, significance set at p=0.05.

	Degrees of freedom	Sum of Squares	F value	P value
Female diet	1	96534	251.0	$<2 \times 10^{-16}$
Clutch diet	1	2623	6.82	0.00914
Female diet*clutch diet	1	13148	34.19	6.68×10^{-9}
Residuals	1053	404991		

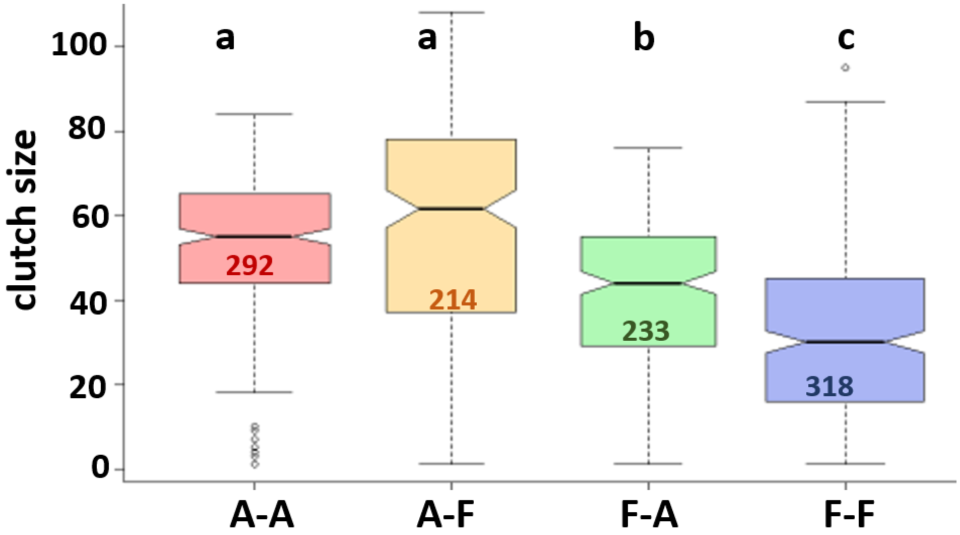

Figure 5: Clutch size according to diet treatment combination (maternal diet - clutch diet, with A=algae, and F=fish food). Boxes represent the first and third quartiles, and notches (or ‘waists’) can be interpreted as confidence intervals. Significant differences are indicated by letters ‘a’ through ‘c’. Sample sizes are shown inside boxes.

When females ate algae, they produced mean clutch sizes of 52.8 ±16.6 and 57.1 ±25.3 when their clutches ate algae and fish food respectively (Fig. 5, A-A and A-F). When females ate fish food, they produced significantly smaller clutches; when clutches ate algae, mean clutch size was 41.3 ±17.0, and when clutches ate fish food, mean clutch size was smallest at 31.2 ±19.7 (Fig. 5, F-A and F-F). Clutch sizes were largest when females, offspring or both ate the live algae diet, whereas clutch sizes were smallest when both they and their mothers ate fish food.

Sex ratio and clutch size both changed over the laying sequence; in the algae-algae treatment, sex ratio was female-biased and clutch sizes were large, whereas in the fish food-fish food treatment, the largest clutches were male-biased (Fig. 6; for simplicity’s sake, only the diet treatments where females and their clutches ate the same food are shown).

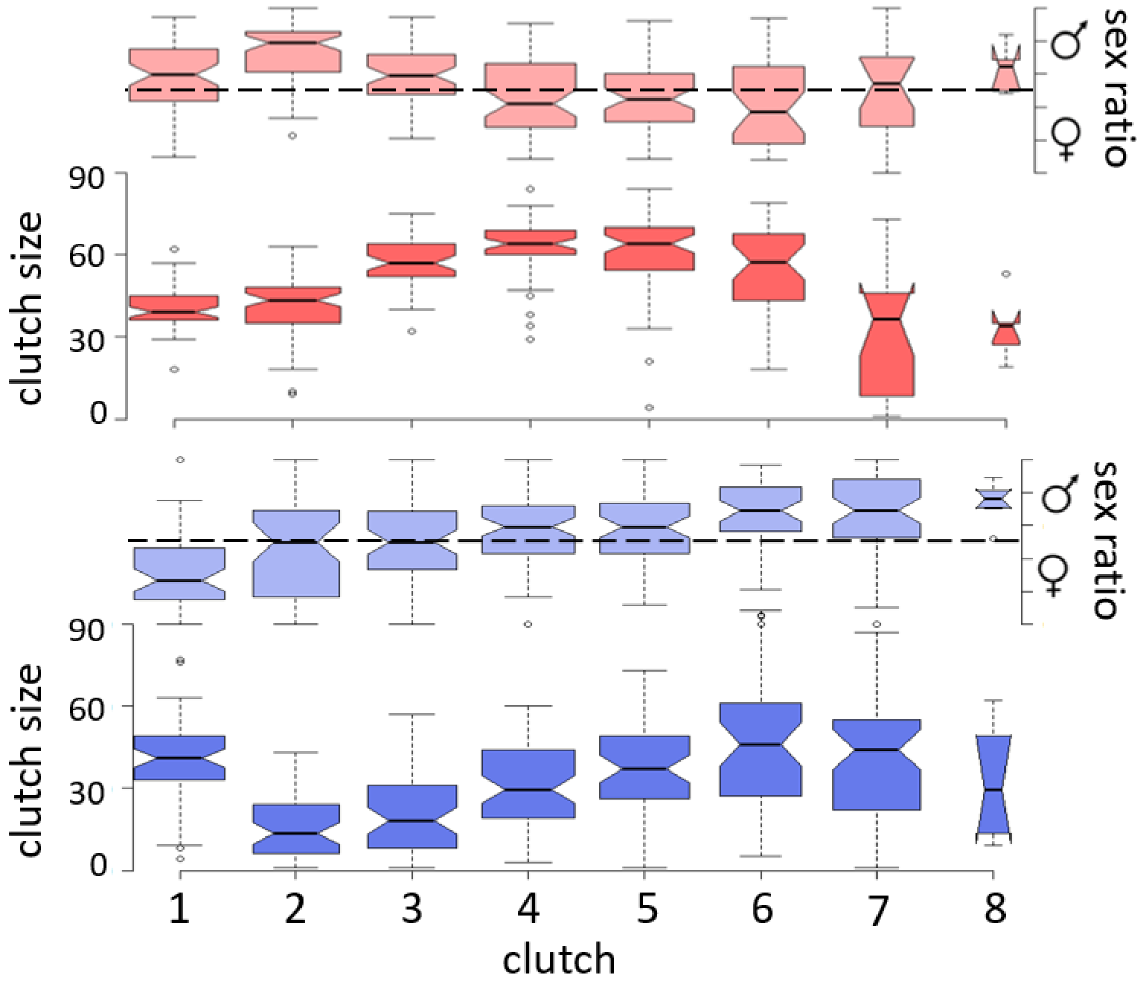

Figure 6: Sex ratio and clutch size plotted against clutch number (parity) for the algae-algae diet combination (top) and the fish food-fish food diet combination (bottom). The dashed line represents the 1:1 sex ratio; below this line is female-biased, and above is male-biased.

The Sex Sequence Analysis

I also ran an additional and separate GLMM analysis, with the same random effect structure (female ID on the intercept) as in the Diet analysis, to distinguish patterns of sex ratio dependence over the sex sequence (=parity, or laying order), independent of the diet manipulation (Ambrosini *et al.* 2014; Table 3). The variables 'AF', 'prevsex', and 'order0' themselves serve to: decouple the first clutch sex ratio from those after it, to indicate how close the focal clutch sex ratio is to the sex ratio of the previous clutch, and to represent clutch laying order (parity) minus 1, respectively (see Ambrosini *et al.* 2014) and Table 3). Pseudo-R^2 was calculated as for other GLMMs, and was ~0.013, explaining about 1% of sex ratio variation. While this Sex Sequence analysis is based on a larger dataset than the Diet analysis, it explains only a tenth of the sex ratio variation than is accounted for by diet in the Diet analysis (1% compared to 11%). Nonetheless, results suggest that the sex ratio of a clutch depends on the sex ratio of the preceding clutch, and do not support a linear sex ratio influence.

Table 3: Candidate model set list, interpretations, and AIC values of the Sex Sequence analysis (GLMMs with binomial errors and a random effect of female ID on the intercept).

Model Structure	Interpretation	DF	AIC	ΔAIC
1. AF + prevsex	Focal clutch sex ratio changes according to the sex ratio of the previous clutch	4	12438.3	0
2. order0 + prevsex	Focal clutch sex ratio changes linearly along the laying sequence and according to the sex ratio of the preceding clutch	4	12568.0	129.7
3. order0	Focal clutch sex ratio changes linearly along the laying sequence	3	12581.1	142.8
4. null	Focal clutch sex ratio is constant throughout the laying sequence	2	12594.7	156.4

Discussion

Sex Ratio and Diet: ESD in *Tigriopus californicus*

The full model that included parity as well as both female and clutch diet (Table 1, #1) explained about 11% more variability in sex ratio than the null (Table 1, #17; Eq. 1). Algae-fed clutches experienced a sex ratio decrease from a strong male bias at about 0.70 to a female bias around 0.40, whereas there was a corresponding increase in sex ratio when clutches ate fish food (Fig. 4). The effect of clutch diet on clutch sex ratio is clear evidence of resource-based environmental sex determination in this species with known polygenic sex determination.

On the genetic side of polygenic sex determination, (Alexander *et al.* 2014) were able to maintain families with male-biased primary sex ratios around 0.70 and female-biased sex ratios around 0.30 (this reflects variance due to genetic factors). On the environmental side of polygenic sex determination (*e.g.* in this study), mean sex ratios fluctuated between roughly 0.40 and 0.70, depending on maternal-offspring diet. Evidently, both genetic and environmental factors influence sex determination to a similar degree in this species.

The resource-based ESD I report in *Tigriopus californicus* is not the first time an environmental factor has been implicated in sex determination in this species. In 1966 Egloff reported that sex ratio increased with temperature by 12% from 15-25°C and proposed that this type of ESD occurred in *T. californicus*; however, Vittor (1971) found no such association. It wasn't until 2002 when (Voordouw and Anholt 2002a) confirmed that while this TSD seemed to be present, they clarified that only about a third of lineages had temperature dependent sex ratios, and the magnitude of the effect was small, on the order of about 5% increase in proportion male from 15-22°C.

I controlled for the effect of temperature in this study by interspersing my diet treatments by blocks in the lab at ambient temperature, so that all treatments were equally exposed to the same temperature regime. For the duration of the experiment, the range of temperature experienced by the copepods was mostly between 18-22°C, with only periodic drops to below 18°C, which is unlikely to have influenced sex ratio much, considering that constant temperatures of 15° and 22° were required for Voordouw and Anholt (2002a) to resolve the temperature effect. I controlled for temperature effects statistically as well: I included female ID as a random effect (modelling the variance due to individual female lineage, including genetic lineage of her and the father, since single paternity is assured), and this would also account for the possibility of temperature sensitivity in her lineage. Visual inspection of the data trends revealed no overall temperature-associated pattern, so I am confident that the sex ratio effect I report here is truly due to diet.

Limited resources and low temperature both influence growth rate, and it has been suggested that it is growth rate rather than temperature or resources that influences sex determination (*e.g.* Takeda 1950 in *T. japonicus*). *Tigriopus japonicus*, like *T.*

californicus, has extremely male-biased sex ratios (Takeda 1950; Egami 1951; Igarashi 1963) and older studies suggested that slowed growth rate (rather than lower temperatures or other factors *per se*) caused lower sex ratios ('increased feminization', *e.g.* Takeda 1950; Egami 1951). More recently, Irigoien *et al.* (2000) suggested that this slowed growth rate/feminization association is a common factor for all copepods. However, Igarashi (1960) found no evidence that slower growth rate caused feminization in *T. japonicus*, and if slowed growth rate caused feminization as supposed, then the high larval density treatment in Voordouw *et al.* (2005) should have shown female-biased sex ratios (since high density was accompanied by nutrient limitation), and this was not the case.

Resources and Sex in *T. californicus*

This study is not the first to investigate the possible link between resources and sex ratio in *T. californicus*. Voordouw *et al*. (2005) investigated sex ratios after experimentally manipulating the body size of females using different larval densities, where females raised at higher larval densities were smaller at maturity because they were resource-limited due to high competition, whereas females at lower densities had plenty of food and grew larger. They found no association between female body size (as a proxy for resource availability) and sex ratio. Because the link between resources and sex ratio was indirect in this case (*i.e.* was achieved via larval density) their approach would not resolve individual level differences in resource allocation, as the design of this study has allowed by tracking sex ratios of individual females. Additionally, Voordouw *et al.* (2005) fed all copepods the same food type, a mix of live cultured *Isochrysis galbana* and TetraMin fishfood flakes (standard lab food = TetraMin fishfood mixture), whereas I fed copepods either *Isochrysis galbana* or TetraMin fish food flakes. Each diet treatment was fed *ad libitum*: the fish food treatment was visually inspected for uneaten food and fed fresh food when necessary, and was consequently never food limited. For the algae diet, I calculated a minimum number of algal cells required (based on Voordouw and Anholt 2002a) and overshot generously. Food limitation is often manifested as smaller clutch sizes in food limited treatments (*e.g. Euterpina acutifrons* - Haq 1972), and the treatment with the smallest clutches in this study was the fish food diet, which was certainly never food limited. I am therefore confident that the difference in sex ratio that I report here is due to a difference in some quality of resources, such as a key nutrient involved in sex development, not quantity of resources overall (that is, the total energy supplied by the two diets was in excess).

The key role of diet quality, not quantity, was emphasized by this study. This indicated that the live *Isochrysis galbana* contained some nutrient that influenced sex ratio, vs. overall amount of nutrients. The identity of this nutrient is unknown: flaked TetraMin fish food is high in most essential nutrients to maintain fish health, and *I. galbana* is considered a high quality algal food for aquaculture (*e.g.* Jalal et al. 2013). The effect of this nutrient on sex ratio might be achieved directly, such as by influencing reproductive hormones or vitellogenesis, or indirectly, through influencing general health or survival.

Differential mortality of the sexes (=larval mortality or naupliar survival) before maturity can bias adult sex ratios, and a key assumption of this study is that the clutch sex ratio, which I measured when the clutches were adults, reflects the primary clutch sex ratio; however, I believe this assumption is met in this study. Firstly, based on past studies on *T. californicus* (*e.g.* Voordouw *et al.* 2005; Voordouw *et al.* 2008) there is no reason to suspect *a priori* that there is differential mortality of the sexes regularly present in the *Tigriopus* system. Secondly, the mean clutch sizes I report are larger than those reported by Vittor (1971) and Egloff (1966), and the maximum clutch size I report is similar to theirs as well (108 in this study, compared to 100, 110, and 140 found by Dybdahl [1994], Vittor [1971], and Egloff [1966] respectively). However, Egloff (1966) and Vittor (1971) both counted eggs (*i.e.* measured actual fecundity), whereas I waited until clutches reached maturity to count offspring as I sexed them. If larval mortality was occurring, this should have resulted in a systematic underestimation of clutch size in my study compared to theirs (since clutch size can only decrease with mortality after oviposition), which is not observed in either the mean clutch sizes or the maximum clutch size. Nonetheless, I compared models based on data corrected for larval mortality (the conservative case; Voordouw and Anholt 2002a; Burris and Dam 2015a), which assigns unsexed juveniles to the rarer sex rather than excluding them from the analysis, to models based on uncorrected data, and I found only trivial differences in model selection and estimates between the two. I elected to present the results from the uncorrected data.

Model Error

One of the drawbacks to a model comparison approach is the potential difficulty in interpreting results; after all, the real world is complex, and more than one factor may interact (Table 1 #1-9), and sex ratio is so variable in this system that potential effect sizes must be large in order to resolve them. To help with this, there are different ways to partition the variance, or error, in a system. I chose generalized linear mixed effects models (GLMMs) to partition variance into two levels, including a random effect of female ID on the intercept. This can be interpreted as the model assigning each female a baseline starting sex ratio (before the fixed effects, *i.e.* diet, are applied) that is distributed normally around the global mean sex ratio (in this case, about 0.56). However, the major statistical caveat to my study is that my GLMMs are over-dispersed, which indicates that a lot of variance was left unexplained by my model (the residual deviance in my GLMMs was about 9x the residual degrees of freedom). Voordouw *et al.* (2005) and other papers from the Anholt lab report residual deviance approximately equal to residual degrees of freedom, but they use simpler GLMs and different experimental designs, which would account for some of the difference between their model fit and mine. A better way to model variance in this study would be to allow each female to produce sex ratios of some value that is distributed normally around her own family's mean sex ratio, which may be different than 0.56, (since we know that family line has an influence on sex ratio); this can be achieved with Bayesian methods and a beta-binomial error distribution. I researched how to use this distribution in R, but at the time of publishing this thesis, there was no package available that could adequately accommodate my data, and learning other statistical analysis software was beyond the scope of my degree. Functionally speaking, a Bayesian analysis would likely widen the confidence intervals in Fig. 4, but would have limited influence on the effect sizes and overall trends the model shows (B. Anholt, pers. comm.), so I interpret my results with cautious confidence.

Stability of PSD in the *Tigriopus* system

Three close congeners of *Tigriopus californicus*, *T. brevicornis*, *T. fulvus*, *T. japonicus*, all have similar mating system with sperm storage (Raisuddin *et al.* 2007), and show similar extra-binomial sex ratio variance to *T. californicus* (*T. fulvus* – Anholt unpub; *T. japonicus* – Igarashi 1963). *Tigriopus japonicus* has cytoplasmic inheritance, wherein sex is inherited like mitochondria (*i.e.* maternal transmission only) so offspring sex ratio is determined by the mothers genotype (Igarashi 1964); however, *T. japonicus* also shows seasonal sex ratio variation, *i.e.* TSD, with no males present in the winter (Egami 1951). *Tigriopus* species also inhabit similar ecological niches (Davenport *et al.* 1997), which suggests the possibility that resource-based environmental sex determination might occur in those systems as well. *Tigriopus* may have speciated relatively recently (Davenport *et al.* 1997), so the similarities and differences between sex determination and sex allocation in the *T. californicus* and *T. japonicus* systems could be informative about the stability of the polygenic system. Polygenic sex determination appears to be stable in *Tigriopus* (Alexander *et al.* 2015; Bateman and Anholt 2017)), as is temperature dependence of sex ratio (*e.g.* Kelly *et al.* 2013; Willett 2010).

Bulmer and Bull (1982) pointed out that if sex ratio heritability under zygotic control is low (as is often the case in chromosomal systems), then populations inhabiting temporally changing environments may experience long-term sex ratio biases because selection is slow to restore the equilibrium sex ratio, which could mean that PSD in *Tigriopus californicus* is a transitionary stage in sex determination. However, I report evidence of a strong environmental effect on sex ratio, in this system as well, and Freedberg (2007) found that fitness gains of producing adaptive sex ratios (*i.e.* sex ratios dependent on an environmental factor) accounted for stability of sex ratio variance in geographically structured populations, so I propose that the sex ratio variance in *T. californicus* is adaptive, that is, dependent on fitness differences between the sexes. This environmental effect could be helping maintain polygenic sex determination against selection for chromosomal/genetic sex determination.

Stable mixed sex determination systems (*i.e.* mixtures of condition-dependent and condition-independent sex factors) have been theoretically hypothesized to be the result of conflict-driven invasions of condition-independent sex factors (*e.g.* sex chromosomes; Kuijper and Pen 2014). Since *Tigriopus* is a system that has a mixture of condition independent (*e.g.* genetic) and condition-dependent (*e.g.* resource-associated environmental) sex factors, and shows strong effects of parent-offspring conflict, this represents an opportunity to empirically test their models.

Although the same authors who report TSD in *Tigriopus californicus* (Voordouw *et al.* 2002a) also confirmed that *T. californicus* had polygenic sex determination (Alexander *et al*. 2015), they did not emphasize that this discovery made *T. californicus* one of only 2 cases of confirmed mixed sex determination systems (including both ESD and GSD) at the time, the other being a lizard with CSD and TSD (Shine *et al.* 2002). Based on results of this study, it seems that sex ratios in *T. californicus* have three distinct contributors to sex ratio variance: temperature-dependent ESD, resource-dependent ESD, and the ~6 sex ratio-associated loci of the polygenic sex determination system. Furthermore, a second lizard with CSD was recently reported to show TSD (Bull 2015), suggesting that mixed sex determination systems may be more common than previously thought.

Resources and Sex Allocation: The 'Costlier' Sex

In the *Tigriopus* system, the females seem to be the sex that requires more resources overall, and there is strong fecundity selection on female body size in copepods in general. Since sex ratio decreased when females and clutches ate algae, I hypothesized based on sex allocation theory that the algae diet was 'better' than the fish food diet, at least with respect to some key nutrient, and that the females needed the 'better' food, because they are the more 'expensive' sex (*i.e.* the sex that benefits the most from parental investment; Trivers and Willard 1973). I expected clutch size to increase with female condition, along with the decrease in sex ratio. I expected clutch sex ratio to decrease for all diet treatments if LRC was occurring since females are the dispersing sex, but the actual pattern of sex ratio change depended on female and clutch diet, which is more consistent with Trivers and Willard-style condition dependence, since diet seemed to influence female condition based on clutch sizes as well as sex ratio.

Clutch sizes confirmed that algae-fed females produced larger clutches, and algae-fed clutches tended to be larger than those fed fish food (though variance in clutch size was higher in the fish food diet than in the algae diet). In this experiment, clutch size reflects the female's fecundity minus larvae who die prior to maturity, and since larval mortality was negligible in this experiment, clutch sizes can be viewed as mostly indicative of female fecundity and condition.

In this study (for clutches 2-8) algae-fed females produced mean clutch sizes of about 52-57, approximately 20% more offspring at the narrowest margin than females who ate fish food and produced clutches of only 31-41 offspring. The smallest mean clutch size in this study was 31 (in the fish food-fish food treatment), larger than all but the largest mean clutch size estimate for *Tigriopus californicus* reported in the wild. The consensus is about 20 eggs/sac (Huizinga 1971; Vittor 1971; Lewis *et al.* 1997), but (Egloff 1966) found a mean brood size of 46 (in the mid-range between my two diet treatments). The largest mean clutch size was algae-fed at 57, comparable to the 58 reported for *T. brevicornis* on a high nutrient diet. Maximum clutch size in this study was 108, comparable to the 100, 110, and 140 reported by Dybdahl (1989), Vittor (1971), and Egloff (1966). Therefore, I am confident that females in this study who ate algae were in better condition than those that did not.

Some of this clutch size variability may be seasonal. There are slightly higher mean clutch sizes in summer, at about 27 eggs/sac (compared to 20 in winter; Lewis *et al.* 1997); the main suggestion for this difference is that food availability is greater in summer, so increased resources are associated with increased clutch sizes. It seems that rather than show decreased clutch size in the 'poorer' fish food diet, *T. californicus* seem instead to do more than twice as well on the 'better' live algae food. This suggests not only that resources are usually limiting in the wild, but also that past lab studies that used fish food as a diet are likely still reflective of average copepod reproductive fitness (rather than reflecting resource-limitation). Harris (1973) and Comita and Comita (1966) report that *Tigriopus brevicornis* has a mean clutch size of 28 and 32 eggs/sac, respectively. Under high food density, the maximum mean brood size reported was 58 eggs/sac (Comita and Comita 1966), nearly the same as the algae-algae treatment in this study (57). The magnitude of difference due to resource quality is therefore similar to the magnitude of difference due to resource quantity.

Note that it is possible for females eating a poorer diet to be in better condition than those eating a higher quality diet: female zebra finches that ate low quality diet were in better condition, possibly because they were forced to lay down stores and the high quality diet females were not (Bradbury and Blakey 1998). These females in poor condition hatched more males, and males are thought to be the more expensive sex (*i.e.* they are the larger sex). In *Tigriopus californicus*, however, this is less likely because eggs represent an expenditure of about 1/3 of the female's biomass (Zurlini, Ferrari, and Nassogne 1978) and they are produced constantly for the entire reproductive life span of the female, presumably precluding much in the way of nutrient reserves. Copepod fatty acids closely track their diet (Brett *et al.* 2009), suggesting the link between condition and diet quality is more direct. (This is also a reason to expect that *T. californicus* should be an income breeder, with their output matching the environmental resources).

For most animals, certain fatty acids are only obtained from the diet (*e.g.* EPA and DHA) and are considered essential nutrients. There is evidence of the role of EPA/DHA in copepod reproduction, so the ratio of specific fatty acids may be important in *T. californicus*. It should be noted that some harpacticoid copepods can facultatively synthesize some fatty acids *de novo* (Nanton and Castell 1998), so the role of certain fatty acids as 'essential' in some copepods is questionable.

There is evidence of resource-based environmental sex determination/sex allocation in two of the major copepod clades: the cyclopoid *Pachypygus gibber* (Becheikh *et al.* 1998), and the harpacticoid *Euterpina acutifrons* (Stancyk and Moreira 1988; Camus and Zeng 2012). Outside of copepods, two invertebrate systems have shown evidence of environmental influence on sex: a polychaete worm *Dinophilus gyrociliatus* and the grasshopper *Cornops aquaticum*.

Resource-based ESD and Sex Allocation in Invertebrates

Pachypygus gibber is a parasitic cyclopoid copepod that has three sex phenotypes, 1 female, 1 small active male, and a second male, big and sedentary. The proportions of these sex phenotypes that develop (*i.e.* sex ratio) depend on the amount of resources, which authors argue is evidence of resource-based ESD (Becheikh *et al.* 1998); authors also report that local sex ratio (in the host) influences a female's sex allocation decision, which suggests some degree of socially mediated sex allocation as well. These features of *P. gibber* resemble those of *T. californicus*, though there are key differences, including the parasitic nature of the former. Becheikh *et al.* (1998) state that local mate competition is at play in *P. gibber* because of their parasitic habit (that is, due to the patchy host habitat and biased local sex ratios within the host). I noted that Becheikh *et al.* (1998) made a key assumption that females must be the more expensive sex, because of their larger size in order to come to their conclusion about resource-based ESD. They did not report the karyotype (chromosome number/type) in this species; I found this surprising, since the presence of sex chromosomes would point to some form of CSD with environmental influence and their absence would suggest polygenic effects on sex determination. I was unable to determine karyotype in *P. gibber*.

Euterpina acutifrons is a planktonic, egg-carrying harpacticoid copepod from the Southern hemisphere which also has three sex phenotypes, 1 female, 1 large male, and 1 small male (both active). The ratio of small males to large males (= male dimorphism) differs between populations and within populations between seasons (Haq 1972; Stancyk and Moreira 1988). Small males are more cold-adapted than large males, and are most abundant in rapidly growing populations, leading to the supposition that male dimorphism is adaptive (Stancyk and Moreira 1988), *i.e.* that the relative proportion of the three sex phenotypes are influenced by some environmental factor. Females store sperm from a single mating to fertilize multiple clutches in sequence, and the average number of egg sacs produced per female tended to increase with the increase in food concentration (Haq 1982), indicating that resources play an important role in *E. acutifrons* reproductive output, as they do in *T. californicus* (this study). Stancyk and Moreira (1988) assert that there is a genetic component to male dimorphism, though authors also suggest that the large male phenotype might be sex-changed females (based on the presence of extremely variable sex ratios which seemed to reflect the productivity of the waters from which they came).

Two decades later Camus and Zeng (2012) looked at *Euterpina acutifrons* naupliar survival and sex ratios on different algal diets: they found that sex ratio was variable and male-biased, and that it was influenced by diet, with fewer females being produced in the worse quality diets (sex ratio was about 0.68 compared to 0.60 on the best diet). They remark that this influence of diet was surprising given that they had previously found sex ratio to be conserved in two other species of copepod; in the calanoid *Acartia sinjiensis* and the paracalanoid *Bestiolina similis*, sex ratio was not significantly affected by photoperiod, stocking density and diets (Camus and Zeng 2012). Authors connect this influence of diet on sex ratio with life history evolution by proposing that the shortened male lifespan is due to males being more sensitive to poor conditions, like low quality food. However, they stop short of recognizing this as a potential clue to the sex determination system in this species of copepod. I propose that taken together, these two papers (Stancyk and Moreira 1988; Camus and Zeng 2012) suggest that sex phenotype in *E. acutifrons*, (whether it be three or two), and therefore sex determination, is dependent on diet. *Tigriopus californicus* sex ratios are variable, male-biased, and even more male-biased on poorer quality diets, and their sex determination system is polygenic, so I looked for information on the karyotype of *E. acutifrons,* though I was unable to learn anything.

A major issue with this interpretation is that Camus and Zeng (2012) don't mention anything about the male dimorphism reported by Stancyk and Moreira (1988), nor do they specify how they sexed the copepods, so it is unclear what is meant by 'sex ratio'. Zurlini *et al*. (1978) reported sex ratio estimated from 10 clutches to be male-biased at 0.60, and also make no mention of male size dimorphism. However, this apparent discord could be explained by the different food types used in the different experiments; Stancyk and Moreira (1988) and Camus and Zeng (2012) used different kinds of algae, and *E. acutifrons* sex ratio (in this case, the proportion of the two male phenotypes) seems to be dependent on diet, as it is in *T. californicus*.

Dinophilus gyrociliatus is a small polychaete worm whose sex ratios and sex determination appear to be influenced by both genetic and environmental factors, as in *T. californicus*. Such factors include age, dietary regime, temperature, salinity and laboratory rearing regime (Prevedelli and Zunarelli Vandini 1999). Females are sex karyotype XX and produce two sizes of eggs; the larger ones become females, and the smaller ones become dwarf males, which are XO. Sex determination therefore is chromosomal and syngamic (= happens upon fertilization; Simonini *et al.* 2003). However, egg provisioning is responsible for the proportion of large eggs relative to small ones (*i.e.* the sex ratio) and this happens progamically (=before fertilization; Simonini *et al.* 2003). Diet quality influences survival, fecundity, and sex ratio: survival was highest on the lowest quality food (cereal – protein deficient), and as in this study, fecundity was greatest on the highest quality food, and sex ratio was male biased on the lowest quality food (Prevedelli and Zunarelli Vandini 1999).

This distinction between progamic and syngamic effects on sex determination and sex allocation illustrates how tightly the primary sex ratio links sex determination and sex allocation. Consider the haplodiploids: the female controls both sex ratio and sex determination syngamically, whereas in *Dinophilus gyrociliatus* the female controls egg provisioning (and sex ratio) but stochastic effects rule the probability of sex chromosome segregation (and sex determination).

Cornops aquaticum is a grasshopper being used as a biocontrol agent for an invasive plant, so Bownes *et al.* (2013) were interested in its fecundity and sex ratios when host plants contained different amounts of foliar resources (nitrogen and phosphorous): they found that females and offspring had higher body weights when eating better quality food (*i.e.* plants with higher foliar nitrogen, keeping carbon content constant); also, females were more fecund and nymphs more likely to survive to adulthood with better food. Most relevantly, sex ratios were female biased on the high quality diet (0.35) and male-biased (0.61) on the low, which is comparable to the magnitude of the dietary effect on *Tigriopus* sex ratio in this study. The authors ensured that total calorie intake was similar, so the effect is based on nutrient quality alone, (that is, the nitrogen and phosphorous ratio).

In the case of *Cornops aquaticum* and *Tigriopus californicus*, juvenile resource provisioning and acquisition played a key role in adult fitness (fecundity and sex ratio respectively) that could not be made up for by later compensation during reproductive adulthood. Bownes *et al.* (2013) pointed out that while fecundity was highest for *C. aquaticum* on highest quality food and lowest on the lowest quality food, when females were switched from a low to a high quality diet, they were able to increase their fecundity but never to the degree that those who had eaten the high quality food throughout their life. In this study, when clutches ate algae, the influence of the mother's diet is small (*i.e.* mothers who ate fish food had clutches with slightly lower sex ratios than those who ate algae at clutch 2, and by clutch 7, the difference was nil; Fig. 4). When clutches ate fish food, however, the influence of the mother's diet was much larger, and remained through clutch 7: clutches whose mothers ate algae had sex ratios about 0.1 lower than those whose mothers ate fish food (Fig. 4). Clutches whose mothers had eaten fish food could not produce sex ratios as low as those whose mothers had eaten algae, which is consistent with the key role of maternal provisioning (and thereby, maternal diet) in *T. californicus* sex allocation.

Based on Bownes *et al.* (2013), I expected that female *C. aquaticum* should be larger than males, because their results suggest that female fitness is more dependent on diet quality (increased nitrogen) than is male fitness; indeed, females are about 30% larger than males (Romero *et al.* 2014), indicating that females are 'more expensive'. Although Bownes *et al.* (2013) reported a dietary effect on sex ratio, and even connected their discussion to the size difference between males and females, they did not suggest that sex determination might be environmental or related to male/female fitness differences, nor do they report karyotype information which would connect their research to the broader relevant sex determination literature. Instead, they propose that their study might be useful to help control the invasive plant better in the future because large females should eat more than smaller males. However, this proposal ignores the fact that they would first have to know how to manipulate the sex determination mechanism in order to influence adult sex ratio to achieve that effect. Romero *et al.* (2014) report that *C. aquaticum* has 22 acrocentric autosomes, and has an XO-XX sex determination system; recall that *Acartia hudsonica* and *Dinophilus gyrogracilis* have this type of chromosomal sex determination as well, and also show evidence of sex ratio dependence (or sex allocation in the case of *A. hudsonica*) on resources.

Resource allocation to current reproductive effort detracts from an individual's future reproductive effort in the trade-off between fecundity and survival (Murdoch 1966), so I expect the survival of female *T. californicus* who ate algae and produced larger clutches to be decreased compared to those who ate fish food and produced smaller clutches. For example, Houslay *et al.* (2015) studied the effect of high and low quality diets in cricket nymphs and adults (in a crossed design like the one used in this study) and found that females that ate a high protein diet as nymphs were very fecund early in life, but had a shorter life, than those that ate a low quality diet as nymphs. The latter lived longer and were most fecund at intermediate lifespans. Similarly, in the planktonic calanoid copepods *Acartia tonsa* and *Acartia hudsonica*, spermatophore production (instead of egg production, analogous to clutch size in this study) is most resource-limited, and declines with age in general, though low early producers compensated by reproducing later into old age (Burris and Dam 2015b). I did not record survival because I sexed up to 8 clutches from each female (out of a possible 15-20 produced over her lifespan), and mortality was very low throughout the experiment, so I cannot gain any insight into female lifetime resource allocation decisions from this study.

Of course, not all invertebrates have sex ratios that depend on the environment. Neither the calanoid copepod *Acartia sinjiensis* nor the paracalanoid *Bestiolina similis* showed sex ratio responses to diet (Camus and Zeng 2012), and as far as I know no one has investigated diet quality on sex ratio in any other *Tigriopus* species. Peterson and Roitberg (2006) found no effect of resource level on sex ratio in the leafcutter bee *Megachile rotundata*, which has variable but male-biased sex ratios of 0.55-0.80 (as reported during use for agriculture pollination). Authors predicted a male-biased sex ratio in lower resource treatments because females are 1.2-1.3 times as big as males (this size-based assumption appears everywhere in sex allocation literature). They found no effect of resource level on sex ratio, although they admit this may be because all of their resource levels were likely limited, confounding the results (Peterson and Roitberg 2006).

Except for the polygenic sex determination in *Tigriopus*, I would say it is unsurprising to find resource-based ESD and resource-based influence on sex ratio and fecundity in *Tigriopus californicus* since it appears to be not uncommon in other invertebrates.

The study of sex allocation has unarguably been fruitful without needing to know the details of the sex determination mechanism (reviews West *et al.* 2000; Rosenfeld and Roberts 2004) and as a new student I was assured that sex allocation theory held regardless of the details of the sex determination mechanism. However, I found that this property of sex allocation theory depends on the assumption that the parent is in control of sex ratio selection. Voordouw and Anholt (2002a) mention that some degree of zygotic selection is expected in systems with ESD and PSD: I think that the resource-based environmental sex determination that I suggest is occurring in *Tigriopus californicus* is evidence of zygotic control of sex determination (and zygotic influence on primary sex ratio), since delayed sex determination until after the period of parental investment frees the offspring from parental sex allocation pressures for a time before its sexual fate is sealed. During this time, the offspring is subject to the environment on its own terms, and can potentially use whichever environmental variable is associated with their greater fitness to influence their own sexual fate, *i.e.* their own sex determination.

Polygenic sex determination is not well studied because it is considered a transitory stage rather than an evolutionarily stable strategy, which violates another assumption of sex allocation theory (that the sex determination mechanism is fixed). For example, sex allocation conclusions (regarding *e.g.* inclusive selection, from Trivers and Hare 1976) rely on parent-offspring and conflict between siblings as key features of the haplodiploids that provided the opportunity for selection to act so strongly on sex allocation, and this required the premise that the genetic architecture of haplodiploids is evolutionarily stable. The *Tigriopus* polygenic system, with its parent-offspring conflict and environmental sex ratio dependence seems to be stable enough to illuminate sex allocation and the evolution and maintenance of sex determination mechanisms.

Chapter 3: Resources and *Tigriopus* Sex Ratios: Carotenoids and Fatty Acids

Overview

While the most striking effect on sex ratio from the Algae experiment (Chapter 2) was due to clutch diet, the female diet did also affect sex ratio and clutch size, and this must have been achieved during the period of parental investment to influence her own fitness. The period of parental investment in *Tigriopus californicus* is during egg provisioning inside the ovaries and during egg ripening in a membranous sac carried on the female's abdomen, and is terminated by hatching. I hypothesized that the live algae and the TetraMin flaked fish food must differ in some key fitness-related nutrient, and that this nutrient would be more closely related to female fitness than male. For my second experiment (the 'Carotene experiment'), I tested whether dietary carotenoids were responsible for the sex ratio effect observed in the Algae experiment by replicating the algae and fish food diet treatments and adding a third, carotenoid-supplemented diet treatment.

Pigmentation and Copepod Fitness

Wild *Tigriopus californicus* are bright orange, and the females carry scarlet ovisacs below their abdomen; this is due to astaxanthin, a red-orange pigment found ubiquitously in crustaceans (Goodwin and Srisukh 1949). Astaxanthin is made from dietary carotenoids, which copepods and other animals cannot synthesize *de novo* (Rhodes 2007; Maoka 2011; Brett *et al.* 2009). In the lab *Tigriopus* turn clear/white when raised on a diet of yeast (Carli *et al.* 1995; Davenport *et al.* 2004) which lacks carotenoids, and a diet containing carotenoids restores colour to unpigmented *Tigriopus brevicornis* (Davenport *et al.* 2004), confirming that dietary carotenoids are required for astaxanthin synthesis in *Tigriopus*. A link between *T. californicus* fitness and algae blooms in the lab was suggested in the observation that lab populations of *T. californicus* seemed to be healthier (more dense and vigorous) than those without algae blooms (Burton and Feldman 1981;

pers. obs.). Keeping the link between dietary carotenoids and astaxanthin content in mind, I investigated the potential role of astaxanthin in *T. californicus* fitness.

Astaxanthin is an extremely effective reactive oxygen species (ROS) scavenger (Rodrigues *et al.* 2012), and reactive oxygen species occur in copepods as a result of two major processes, UV irradiation and lipid metabolism (Caramujo *et al.* 2012; Lotocka and Styczyńska-Jurewicz 2001; Juhl *et al.* 1996). *Tigriopus californicus* are often exposed to high amounts of ultraviolet radiation from which they have no refuge. During summer months especially, pools are usually very shallow and unshaded (Martin *et al.* 2000); *Tigriopus* preferred an artificial UV refuge at midday when it was offered, however, a behavioural trait indicative of the potential of UV radiation as a selective agent (Martin *et al.* 2000).

Tigriopus californicus is about twice as resistant to UV irradiation as planktonic copepods like *Acartia clausii*, and this has been attributed to the greater astaxanthin content in *T. californicus* adults, the shallow nature of their pools and lack of UV refuge (Chalker-Scott 1995), because pigmented copepods are less susceptible to UV radiation (Caramujo *et al.* 2012; Chalker-Scott 1995). Eggs are as tolerant as adults to UV irradiation (Kane and Pomory 2001), possibly because female *Tigriopus* allocate about half their total body content of astaxanthin to their eggs (Goodwin and Srisukh 1949), which makes the ovisac a striking bright red colour when ripe (*i.e.* nearing hatching). Since nauplii have not been observed feeding in N-I and N-II stages (Lewis *et al.* 1997) and must therefore be subsisting off of stores from the yolk, all their astaxanthin must be allocated to them by their mother during egg provisioning.

In wild *Diaptomus* (freshwater planktonic copepod), a colour polymorphism between lakes was the result of a differential ability to uptake carotenoids, and that this seemed to be maintained by the selective trade-off between UV and predation (more astaxanthin = better UV protection, but also more visibility to predators; Hairston 1979). In the lab, a yeast diet was associated with differences in *Tigriopus* reproduction compared to a live algae (*Pavlova lutheri*) with higher naupliar survival on the yeast diet, whereas fecundity was higher on the algae diet (Carli *et al.* 1995). *Tigriopus japonicus* who were given different diets, including *Actinobacter* bacteria, yeast (both lacking carotenoids), blue-green alga *Chlorella*, or green alga *Chlorococcum* (both containing carotenoids) were most productive when eating the algae and bacteria together, but not bacteria alone (Lee 1991), consistent with a role for dietary pigment in reproduction. It seems likely that there would be fitness benefits for *Tigriopus californicus* that could be mediated by astaxanthin through the selective influence of UV radiation, as in (Kane and Pomory 2001; Hairston 1979; Davenport *et al.* 2004; Caramujo *et al.* 2012).

There is some evidence that astaxanthin might be directly linked with sex ratio as well. First, Chalker-Scott (1995) found that clutches at the N-I and N-II stage that were irradiated with UV were more male-biased, suggesting a possible link between UV (and by extension, astaxanthin) and sex ratio. Second, Igarashi (1963) reported that the colour of the egg sac in *T. japonicus* was the best predictor of sex ratio: there are two types of ripe sacs, whiteish ones (male-biased) which gleamed under UV light, and blueish ones (female biased) which did not, and this is a continuum. Although I learned that the colour of the egg sac during ripening is due to astaxanthin being freed from its protein bonds (Cianci *et al.* 2002), I knew from my own experiments that Igarashi (1963)'s observation in *T. japonicus* resembled my own in *T. californicus*. Lab-raised (and presumably carotene/astaxanthin-limited) and wild copepods produced ovisacs that showed different colour change (ripening) patterns: unripe lab-raised egg sacs were overall bluer (compared to green in wild ones) and ripe egg sacs were more colourless/clear (compared to translucent orange with a bright red spot; pers. obs.). If ovisac colour was variable and associated with sex ratio in *T. japonicus*, and *T. californicus* shows similar ovisac colour variability, then sex ratio may be associated with ovisac colour (and therefore astaxanthin) in *T. californicus* as well.

Astaxanthin may also be related to *Tigriopus californicus* fitness through lipid metabolism which also produces reactive oxygen species (Lotocka and Styczyńska-Jurewicz 2001; Juhl *et al.* 1996). Astaxanthin esters increase at key points in *Acartia* development, which suggest its role in lipid metabolism an ROS scavenger (Lotocka and Styczyńska-Jurewicz 2001). The *T. californicus* N-I and N-II nauplii do not seem to feed (Lewis *et al.* 1997) but have high growth rates (Harris 1973), and presumably are metabolizing lipid stores from the yolk; during this stage, astaxanthin content may limit their ability for rapid growth (Lotocka and Styczyńska-Jurewicz 2001). Eggs of the red devil (*Cichlasoma citrinellum,* a fish) had the greatest relative concentration of astaxanthin in the body (Pan and Chien 2009); since fish eggs are not exposed to much UV radiation, being protected under water (this is consistent with a role for astaxanthin in fish fitness independent of the influence of UV radiation). A slower growth rate has been hypothesized to cause feminization in *Tigriopus japonicus* (Egami 1951), and Irigoien *et al.* (2000) proposed that growth rate and sex ratio were linked in all copepods. If astaxanthin availability can limit growth at key points in development, and growth rate influences sex, astaxanthin (and dietary carotenoids) may be associated with *T. californicus* sex ratio.

Different algae species have different concentrations of carotenoids (Juhl *et al.* 1996), and even the same algae species can have different concentrations of carotenoids (Jalal *et al.* 2013) depending on extrinsic (*e.g.* temperature, nutrient levels) and intrinsic (*e.g.* strain, species) factors, so dietary carotenoids could be considered a patchily distributed resource (Lee and Taga 1988). For example, (Egloff 1966) sampled chlorophyll a in tidepools containing *T. californicus* and found sex ratio decreased (*i.e.* there were more females) with increasing chlorophyll a; since chlorophyll a is positively correlated to carotenoid content (Datt 1998), increased carotenoids were also associated with decreased sex ratio. Furthermore, astaxanthin expenditure is an expense borne by females alone, since they alone contribute to egg provisioning (Goodwin and Srisukh 1949), indicative of a potential for strong sex-differential selection around astaxanthin/dietary carotenoid availability.

TetraMin fish food was used in experiments reported in this thesis as well as in many other lab studies on *Tigriopus californicus* (*e.g.* Voordouw and Anholt 2002a; Voordouw *et al.* 2005; Voordouw *et al.* 2008; Alexander *et al.* 2015; Tai 2014; Foley *et al.* 2011), since it is convenient, and copepods can be raised for many generations (14 in the Anholt lab) without suffering population collapse. It contains vitamin A acetate (Fig. 7), which is about half a β-carotene molecule, whereas the live algae itself contains the full molecule (along with other carotenoids). Since astaxanthin uptake and function is strongly dependent on its molecular form (Ambati *et al.* 2014; Rodrigues *et al.* 2012), it is reasonable to assume that its synthesis requires specific molecules in a useable format, and that the vitamin A acetate might not be sufficiently close in form to β-carotene for the copepod to synthesize sufficient astaxanthin when eating a strictly fish food diet compared to a live algae diet. This deficiency in dietary carotenoids (and astaxanthin) may be responsible for the diet-dependent sex ratios I report in the Algae experiment.

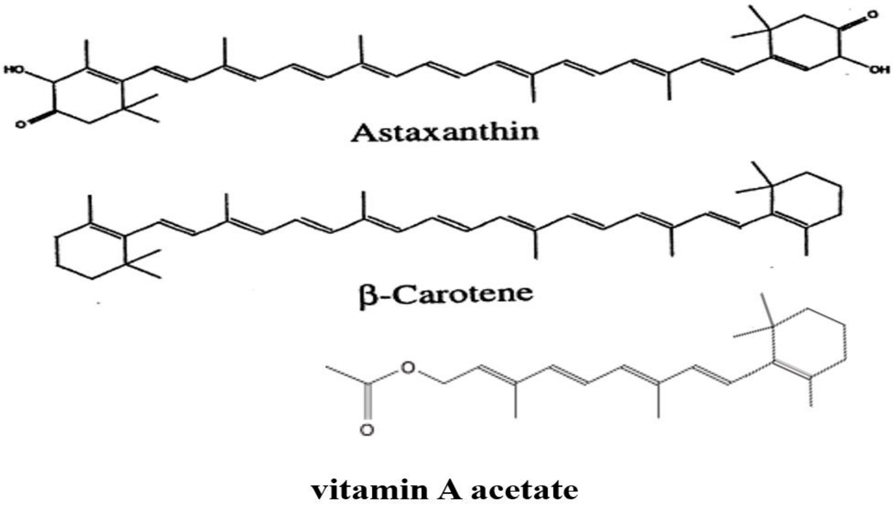

Figure 7: Three pigment molecules potentially involved in sex allocation in *T. californicus;* astaxanthin is the molecule present in animals, whereas β-carotene is present in the algae. Vitamin A acetate is the closest relevant molecule present in flaked TetraMin fish food.

To decisively test whether dietary carotenoids were associated with sex ratios in *Tigriopus californicus*, I re-ran the Algae Experiment with an extra diet treatment. I supplemented plain TetraMin fish food with β-carotene, to compare it to the plain fishfood and live algae-fed diet treatments, and I did this with females and clutches, resulting in 9 female-offspring diet combinations (A-A, A-F, A-C, F-A, F-F, F-C, C-A, C-F, C-C, where A=algae, F=fish food, and C=carotene-supplemented fish food). I expected that if β-carotene (and therefore astaxanthin) was implicated in the dietary sex ratio effect, then female fitness was more likely to be improved than male, and the carotene-supplemented diet should be associated with female-biased sex ratios similar to the live algae diet (which of course also has carotenoids). However, if carotenoids/astaxanthin are not linked to sex ratio, then sex ratios of copepods fed the carotene-supplemented diet should be like those who ate the plain fish food diet.

A secondary objective of this experiment was to replicate the sex ratio effect caused by the algae and fish food diets in the Algae experiment; accordingly, I expected the live algae to be associated with increased clutch sizes and decreased sex ratios (more females) compared to the fish food diet which should show smaller clutch sizes and increased sex ratios (more males).

Methods

This experiment was similar in design to the Algae Experiment, with two major differences. Instead of selecting already mature females of unknown ages for use the in the experiment, I selected clasped pairs, to ensure that the females were all late-stage juveniles entering their respective diet treatments. I hoped that this would help resolve an effect of female age on sex ratio, if it was present. Secondly, shellfish aquaculture facility Nova Harvest provided me with me live T-ISO (a Tahitian clone of *Isochrysis* aff. *galbana*), cultured either in an automated bioreactor or in columns or bags; saving me the job of regular batch-culturing that was necessary for the Algae experiment.

I collected wild *Tigriopus californicus* from Aguilar Beach, Bamfield, BC, Canada (48° 50' 10.8708" N 125° 8' 35.1852" W) in late February 2016. Copepods acclimated for one night in the lab. I indiscriminately selected 324 pairs of copepods (males clasping C4-C5 females – males were removed from the experiment after insemination) and placed 108 pairs in each of three diet treatments: algae (A - live T-ISO), fish food (F – TetraMin™ fishfood mixture, as in the Algae Experiment) or carotene (C – TetraMin™ fishfood mixture, plus 10µg β-carotene). I observed females for the next month in their individual 60mL wells, removed a maximum of 6 ovisacs in sequence as they ripened (limited to 6 by time and resources), and raised the resulting clutches to maturity (each clutch in its own 60mL well) eating their respective diet treatment (algae, fish food, or carotene; 36 females for each of 9 treatment combinations: female-clutch: A-A, A-F, A-C, F-A, F-F, F-C, C-A, C-F, C-C). I sexed these clutches once they were sexually mature and sexually dimorphic and recorded the number of males, females, and juveniles, as in the Algae Experiment, to derive the clutch sex ratio (= males / [males + females]) and clutch size (= males + females + juveniles).

Diet Regimes

There were three diet regimes in this experiment: algae, fish food, and carotene supplemented fish food (or just ‘carotene’). The algae diet consisted of live T-ISO cells (*Isochrysis* aff. *galbana* clone, NCMA CCMP1324), which were cultured by Nova

Harvest Aquaculture Facility (Bamfield BC) in modified Walnes algal medium. Approximate cell density counts were recorded daily, and copepods were fed so that suspended algal cells were always present. The fish food diet was the same as in the Algae Experiment: a slurry was made from 0.2g TetraMin™ Tropical Flakes, 0.2g Omega One™ Super Veggie Kelp Flakes, and 40mL filtered salt water. Fish food and carotene clutches were fed 30-60mL whenever they ran out of food (usually ~3days) based on visual inspection. Walnes algal medium was added to control for micronutrient differences due to the medium rather than the algae. The carotene diet is the same as the fish food diet, but includes 10µg β-carotene (≥93% pure, Sigma Aldritch Co., Lot#MKBQ6333V) as well. Beta-carotene is not soluble in water, so it was necessary to grind it into the fish food with a pestle, to ensure that it adhered to the lipids so that it could be suspended in sea water with the rest of the food (astaxanthin and β-carotene are taken up best with oils, since they are lipid soluble; Ambati *et al.* 2014). It was also necessary to carry out a pigment extraction and quantification to confirm whether the copepods could uptake and metabolize the synthetic β-carotene into astaxanthin (see Appendix A: Pigment Analysis).

Penstrep™ (antimicrobial penicillin-streptomycin) was added to clutches in all diet treatments as in the Algae Experiment, because early naupliar stages are susceptible to mortality from bacterial or fungal growth.

Temperature

Experimental females and their clutches, with each individual female and each clutch in 60mL wells, were interspersed in blocks on shelves in an incubator set at 20°C with a 12:12 light: dark period, with the expectation that the incubator would minimize temperature variation, compared to ambient temperature on shelves in the lab (as in the Algae Experiment). All treatments were regularly removed for feeding and replaced on different shelves every 2-3 days. While the incubator became fouled with ice near the end of the experiment, causing delayed growth in some clutch 6 samples, I found no pattern between sex ratio or clutch size and temperature in this study, using results of the temperature log (using iButtons, as in the Algae Experiment).

Statistical Analysis

As in the Algae Experiment, I used a model comparison approach. I modeled sex ratios using GLMMs, and chose the best model from a nested candidate set which included three predictor variables (female diet, clutch diet, and parity) and a random effect on the intercept (female ID), as in the Algae Experiment

To estimate the explanatory value of the best model, I used the same formula as in the Algae Experiment:

$$\text{pseudo-}R^2 = 1 - (\text{log likelihood[best model]} / \text{log likelihood[null model]})$$

I also investigated total clutch size, as in the Algae Experiment, using GLMMs and ANOVA; only the ANOVA and Tukey posthoc results are presented for simplicity.

Results

The best model based on AIC included all three explanatory variables and their interactions (clutch diet*parity* clutch diet -Table 4); this model had a pseudo-R^2 value of 0.0303, indicating that my explanatory variables together with the random effect explain about 3% of the total variation in sex ratio.

Table 4: Candidate model set of GLMM structures and AIC information of the effect of female and clutch diet and laying order (parity) on clutch sex ratio; all models have binomial errors and a random effect on female ID.

Model Structure	DF	AIC	ΔAIC
clutch diet * parity * female diet	19	9378.0	0
clutch diet * parity + female diet	9	9443.7	65.7
clutch diet * parity	7	9445.5	67.5
parity * female diet + clutch diet	9	9450.5	72.6
parity * female diet	7	9455.1	77.1
clutch diet + female diet + parity	7	9503.2	125.3
clutch diet + parity	5	9504.9	127.0
parity + female diet	5	9507.4	129.5
parity	3	9508.8	130.9
clutch diet * female diet + parity	11	9508.9	131.0
female diet + clutch diet	6	9630.1	252.2
female diet	4	9632.8	254.9
clutch diet	4	9633.5	255.6
clutch diet*female diet	10	9635.3	257.3
null	2	9635.9	257.9

For the purpose of this thesis a simplified figure (Figure 9) suffices, which shows the three treatments where females and offspring ate the same diet. The fish food (-fish food) and carotene (-carotene) diet treatments have similar sex ratios regardless of parity (~0.50-0.55), though the algae (-algae) treatment started with a sex ratio of 0.32 in clutch 1 which increased to 0.45 in clutch 6. Other diet treatment combinations show similar trends.

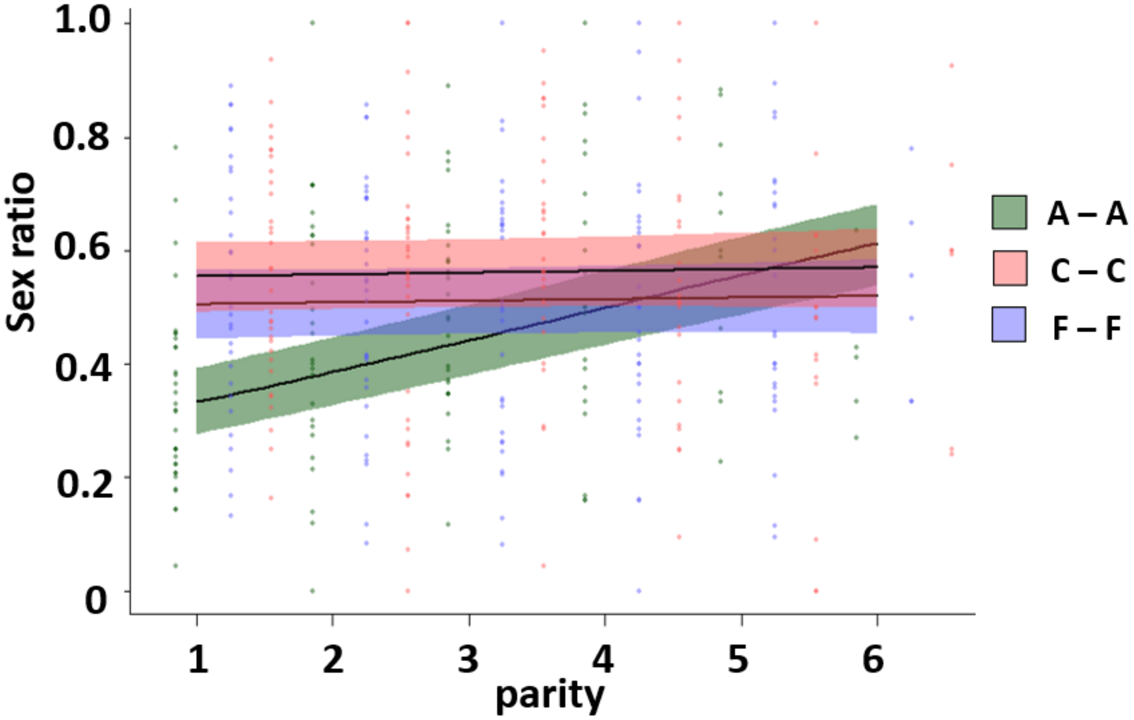

Figure 8: Sex ratios of three diet combinations where females and clutches ate the same diet (only 3 of the 9 are plotted for simplicity, though they share similar patterns). Legend specifies algae (A), fishfood (F) and carotene supplemented (C) diets, in order 'female diet – clutch diet'. The full GLMM model included female diet, clutch diet, and parity, and their three-way interaction. Observed sex ratios are plotted in corresponding colours behind the model predictions (black lines) and shaded polygons represent confidence bounds (based on posthoc best linear unbiased predictors).

Clutch size was largely similar between the fish food and carotene treatments regardless of parity. However, when females ate algae, their clutch size was low, with a mean of 35 (SD=22) compared to the fish food and carotene treatments, with means of 49 (23) and 50 (27) respectively. However, when offspring ate algae themselves, clutch sizes were larger than fish food and carotene treatments, with means of 55 (22) compared to 39 (25) and 41 (25), respectively.

Table 5: Analysis of clutch size variance due to female diet and clutch diet; significance was set at p=0.05.

	DF	Sum of Squares	F value	P value
Female diet	2	49778	47.84	$<2 \times 10^{-16}$
Clutch diet	2	69959	67.24	$<2 \times 10^{-16}$
Female diet*clutch diet	4	20302	9.76	9.0×10^{-8}
Residuals	1247	648732		

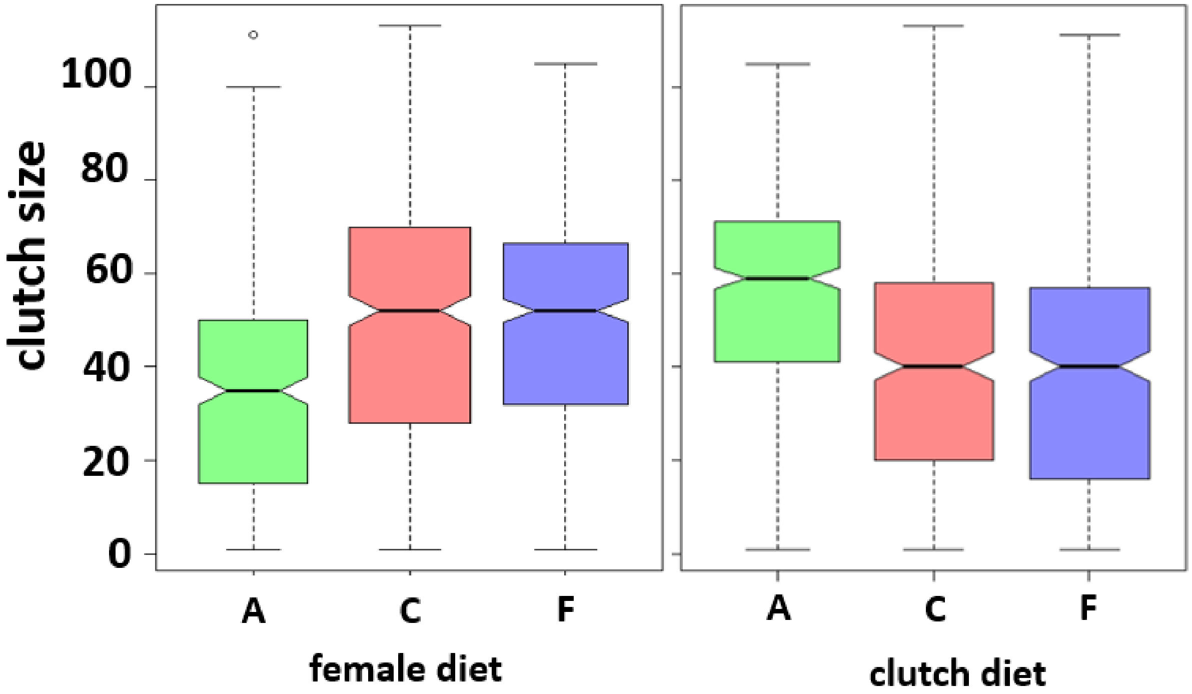

Figure 9: Clutch sizes plotted against (left) female diet, with clutch diet signified by A (algae), C (carotene-supplemented fish food) and F (fish food); on the right, clutch diet, with female diet signified by A, C, or F.

Discussion

Carotenoid Supplementation and Sex Ratio in *T. californicus*

I hypothesized that increased dietary carotenoids in the form of supplemented β-carotene would increase astaxanthin concentration in *Tigriopus californicus* and that these copepods would produce more daughters than those that had not been supplemented. Although the β-carotene supplementation did increase astaxanthin content in the copepods eating the carotene-supplemented diet treatment (Appendix A), I found that the sex ratios of clutches produced by females eating the carotene-supplemented (*i.e.* 'carotene') diet and the fish food diet were similar (that is, the model predictions each lie within the other's confidence intervals; C-C and F-F in Fig. 8). In the carotene and fish food diet treatments, sex ratio did not change with parity but remained at ~ 0.50 – 0.55 over six clutches, whereas sex ratio in the algae diet increased from 0.32 to 0.45 from clutch 1 to 6 (Fig. 8).

The carotene and fish food mixtures themselves were identical except in β-carotene content (see Methods), so I used a pigment analysis (Appendix A) to confirm that the copepods were able to uptake and metabolize it. The same copepods that ate each of the three diets from this experiment were sacrificed at the end of the experiment so I could extract and quantify the astaxanthin present in body tissues. I expected that carotene-supplemented copepods should have higher astaxanthin content than the fish food-fed (unsupplemented) copepods if the experimental manipulation was successful. I found that females especially showed higher astaxanthin content in the carotene diet than in the fish food diet (though the trend was consistent for males as well), confirming that copepods could uptake and metabolize the β-carotene into astaxanthin. Nonetheless, sex ratios produced by copepods in the fish food and carotene diet treatments were more similar to each other than to the algae diet, even though the astaxanthin content (which reflects the carotenoids available in the diet) was more similar between the fishfood and algae diet. This is further support that astaxanthin and sex ratio are not linked in *Tigriopus californicus*. I conclude that while carotene supplementation seemed to be successful, it had no effect on sex ratio; it is unlikely that dietary carotenoids (astaxanthin) are the patchily distributed, fitness-associated resource responsible for sex ratio selection in *T. californicus*.

I was unable to find any other explicit test of the effect of carotenoids or astaxanthin on sex ratio in any copepod, however this null result is consistent with some patterns reported elsewhere. *Tigriopus fulvus* can reproduce on a diet of yeast or *Monochrysis* (=*Pavlova*) *lutheri* , but many algae-fed females were infertile (Carli *et al.* 1995) whereas yeast-fed copepods showed smaller but more consistent naupliar production. Their yeast-fed copepods are analogous to my fish food-fed copepods (lacking dietary carotenoids), and in their study as in this study, an algae diet was not associated with larger clutch sizes, and not consistent with carotenoids being associated with female reproductive success. In the field, egg production in *Calanus helgolandicus* was not correlated with plankton biomass in the field, or the concentrations of chlorophyll a or diatoms (Poulet *et al.* 2006). Similarly Arendt *et al.*(2005) found no association between egg production and chlorophyll a in *Temora longicornis*. Though Egloff (1966) reported decreasing sex ratio with increasing chlorophyll a, he also found that tidepool chlorophyll a levels were 10-100x higher than those in open water. It is likely that carotenoids are not a limiting resource in the *Tigriopus* system, and therefore may not be important for selection on sex ratio. This study, which found no effect of β-carotene supplementation on *Tigriopus californicus* sex ratio or clutch size, is consistent with these.

Live Algae and Sex Ratio in *T. californicus*

This experiment was also an opportunity to replicate the Algae experiment, in which algae-fed copepods showed decreasing sex ratio (increasing female bias), and clutch size was larger in algae-fed diet treatments. Accordingly, I expected sex ratio to decrease over the laying order (=parity) when females and clutches ate live algae. However, a different pattern was observed in the Carotene experiment. There was still an effect of live algae diet on sex ratio (compared to sex ratios in the fish food treatment, which did not change), but sex ratio increased from 0.32 in clutch 1 to 0.45 in clutch 6, rather than decreasing from about 0.70 to about 0.40 as in the Algae experiment. Furthermore, females eating the algae diet showed smaller clutch sizes as the experiment progressed, and many stopped producing egg sacs at all by the fourth clutch. This is reflected in the mean clutch sizes when plotted according to maternal diet, which were ~30% smaller when females ate algae than when they ate fish food or carotene diets (35, compared to 49 and 50 respectively; Fig. 8). In contrast, clutch sizes were about 25% larger when clutches ate algae compared to those who ate fish food or carotene (55, compared to 39 and 41 respectively; Fig. 8), which implies that the T-ISO live algae used in this experiment lacked some key nutrient (or perhaps contained a toxin) that specifically influences reproduction (*i.e.* female fitness). This is especially surprising since live T-ISO does not produce anti-feedants and is well-known to be high quality food for aquaculture (*e.g.* Sukenik and Wahnon 1991).

One possible reason for the disparity between the two experiments may be female age. All females in the carotene experiment were young, and the first clutch I recorded was the first clutch they produced. On the other hand, I do not know the age of females in the first experiment, as they were collected in the wild (though mortality was low). Females fed a fish food-fish food diet in the Carotene Experiment showed no sex ratio change with parity, but in the Algae Experiment showed increasing sex ratios, and this observation is puzzling. Perhaps *Tigriopus* females produce male-biased sex ratios during the first portion of their adult life, and female-biased sex ratios towards the end of their life, but this pattern is mostly visible in algae-fed copepods. Another possibility is that diet and age interact, and this study was not able to resolve age effects. I did not conduct a sex sequence analysis (as I did in the Algae Experiment), which is better equipped to resolve linear age effects (see p. 19), because I judged my sample size too small to produce meaningful output.

While a diet-by-age interaction effect on sex ratio cannot be ruled out, the disparity between sex ratio trends when copepods were fed live algae in the Algae experiment and live algae in the Carotene experiment suggests that the nutrient causing sex ratio variation in *Tigriopus californicus* is a feature of live algae itself.. Nutrient content in algae can vary widely depending on strain and culture conditions (*e.g.* Molina Grima *et al.* 1992; Jalal *et al.* 2013; Huerlimann *et al.* 2010). It has been known for a long time that closely related algae species may differ in their nutritional suitability for maintaining crustacean reproduction in the lab: for *Daphnia, Scenedesmus spinosus* is an excellent food, but *S. oahuensis* and *S. quadricauda* are mediocre or poor; also, *Platymonas* strain No. 5

supports only 2 generations of *Tigriopus californicus* but 8 of *T. japonicus* (Provasoli *et al.* 1959). Both strain and culture rearing conditions differed between the Algae and Carotene Experiments.

In the Algae Experiment, I used *I. galbana* Parke (strain identifier CCMP1323), and since it was associated with larger clutch sizes and more females, it seemed to be very good quality food for *T. californicus*. In the Carotene Experiment, I used *Isochrysis* aff. *galbana*, called T-ISO (strain identifier CCMP1324), a Tahitian clone strain commonly used in aquaculture, and this algae was associated with decreased clutch sizes and more males, and seemed to be poor quality food for reproduction (that is – producing eggs) though adequate for growth of juveniles to adulthood. T-ISO is capable of adapting to a much broader range of temperature/light conditions than *I. galbana* and has an optimum growth temperature of 27.5 °C, whereas *I. galbana* shows no growth at all at 27.5 °C, but grows best near 20 °C (Ewart and Pruder 1981). Type and proportion of fatty acids, which range in length from 10 to 24 carbons, are often similar between algae species of the same taxon but differ greatly between taxa (Hu *et al.* 2008). While the total lipid content of *I. galbana* and T-ISO are similar, at about 20-23% dry weight (Whyte 1987; Helm and Laing 1987), relative proportions of polyunsaturated fatty acids (PUFA), specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), seem to differ between the two strains, though it is difficult to say for sure because of the widespread habit of researchers not reporting which strain was used (beyond simply "*Isochrysis galbana*"; Molina-Grima *et al.* 1992); contamination or mislabelling is also a possible issue. Nonetheless, one comparison of the fatty acid profiles in T-ISO and *I. galbana* reported that T-ISO lacks EPA, while DHA is present in both (Helm and Laing 1987). Another study tested multiple strains of *Isochrysis* (including T-ISO, but not *I. galbana*) and found that all strains had almost the same fatty acid composition, and contained DHA, but not EPA (Liu *et al*. 2013); however, although this study included T-ISO, *I. galbana* was not tested because it grew too slowly. Evidence suggests that T-ISO contains DHA, but not EPA, whereas *I. galbana* contains both EPA and DHA.

Culture and rearing conditions also affect nutrient content in algae. As a culture ages, lipid content generally increases from early to late logarithmic growth phase, with highest yields in late logarithmic phase and at the beginning of the stationary phase; furthermore, lipids of the logarithmic phase are polar membrane lipids, whereas in the stationary phase, these are converted to neutral storage lipids (Huerlimann *et al.* 2010). In the Algae Experiment, I hand-raised batch monocultures of *I. galbana* in 500mL flasks, and harvested algae on the 7-14th days of its growth cycle (the late logarithmic and early stationary phases), when it had maximum nutritional value. In the Carotene Experiment, Nova Harvest Aquaculture supplied me with T-ISO that had been continuously cultured either by bag or column: algae cultured in bags were supplied with nutrients throughout the growth cycle, whereas algae grown in columns were provided with all nutrients at the start of the growth cycle (K. Rollheiser, pers. comm.). Consequently, although I am certain the T-ISO supplied to me was nutritionally complete for general invertebrate growth (since Nova Harvest fed its oysters the same algae and reported regular growth), this T-ISO may have differed from *I. galbana* in that it was deficient in a key nutrient (such as the fatty acid EPA), or overall of more variable (lower) nutritional quality. Unlike many other algae, neither T-ISO nor *I. galbana* are known to produce antifeedant toxins (Sukenik and Wahnon 1991).

Copepod Reproduction and Fatty Acids

Many studies have linked copepod reproduction (egg production rate/fecundity, hatching success/egg inviability) and fatty acids (*e.g.* Arendt *et al.* 2005; Lacoste *et al.* 2001; Evjemo *et al.* 2008; Poulet *et al.* 2006, Poulet *et al*. 2007; Irigoien *et al.* 2000). Polyunsaturated fatty acids (PUFA), especially DHA and EPA, are distributed heterogeneously in the environment; diatoms, which occur in spring/summer and contain high amounts of PUFA (including both EPA and DHA), and produce anti-feedants that can inhibit reproduction, whereas dinoflagellates, which occur in autumn and winter, contain lower amounts of PUFA (which is mostly DHA), but do not produce such toxins (Vargas *et al.* 2006; Jónasdóttir *et al.* 2009; Evjemo *et al.* 2008). It is difficult to distinguish dietary effects on copepod reproduction due to fatty acids from those due to antifeedants, especially since the same species that produce more EPA often produce antifeedants, and these are expected to have opposing effects on copepod fitness (Guisande *et al*. 2000; Jónasdóttir *et al.* 2009; Evjemo *et al.* 2008).

According to Poulet *et al.* (2007), the morphology of gonads, oocyte maturation and variation of egg production rate are all closely linked. In all experiments that I have conducted with *Tigriopus californicus*, I have observed ovary and ovisac abnormalities, particularly when copepods were fed fish food diets. In the Carotene experiment, females fed T-ISO showed decreasing clutch sizes and many stopped producing clutches at all around their fourth clutch. This pattern resembles those reported by Lacoste *et al*. (2001) and Poulet *et al.* (2007) in the calanoid copepod *Calanus helgolandicus*, where failure to reproduce was caused by atresia, or degeneration of the ovary. Irreversible sterilization occurred when females ate the chlorophyte algae *Dunaliella*, which is missing two essential fatty acids, EPA and DHA (Lacoste *et al.* 2001).

There is evidence that maternal reserves of EPA are involved in egg production in *Temora longicornis* (Jonasdottir *et al.* 2009), and that female copepods actively allocate fatty acids to their eggs (Brett *et al.* 2009)**.** This represents a possible mechanism by which female copepods might skew sex ratio through egg provisioning (if, say, eggs are more likely to become female if they have a higher proportion of EPA/DHA).

Euterpina acutifrons is a harpacticoid, egg-carrying copepod like *T. californicus* that shows evidence of a link between sex ratio and diet. As in this study, decreased larval survival and egg production were associated with a T-ISO diet (compared to three other algal diets; Camus and Zeng 2012). Furthermore, sex ratio was more male-biased on T-ISO at 0.68, than on the best diet, at 0.60, suggesting that sex ratio and clutch size may linked through dietary fatty acids. However, it is unclear what ‘sex ratio’ is being reported since the Camus and Zeng (2012) study does not mention that *E. acutifrons* has two male morphs (Haq 1973; Stancyk and Moreira 1988). However, Camus and Zeng (2012) fed copepods *I. galbana*, whereas Stancyk and Moreira (1988) used T-ISO. Since it seems this strain difference may be related to sex ratio in *T. californicus*, I suggest that it might have influenced the male dimorphism (sex ratio) in *E. acutifrons* as well, and explain why only some authors have reported male dimorphism in this species. I could not find any information on sex determination mechanisms or karyotype in *E. acutifrons*.

Irigoien *et al.* (2000) looked at sex ratios of *Calanus helgolandicus* on three algal diets, representing the major phytoplankton prey groups in the ocean: flagellates (*Isochrysis galbana,* strain not reported), dinoflagellates (*Prorocentrum micans)*, and diatoms

(*Thalassiosira weissflogii*) and found increased food concentration associated with increasing sex ratio (more males); the authors frame their study around the hypothesis that differing growth rates influence sex ratio. However, the prey groups used by Irigoien *et al.* (2000) represent diversity in fatty acids (especially EPA and DHA), and lipid metabolism is tightly linked to growth rate (Brett *et al.* 2009), so it may be that sex ratios were influenced by diet in an unexpected way. Irigoien *et al.* (2000) conclude that both genetic and environmental sex determination may exist in this species, as in the parasitic copepod *Pachypygus gibber* (Becheikh *et al*. 1998).

If EPA is truly necessary for female *Tigriopus californicus* reproduction, it is surprising that populations could be raised so successfully in the lab for so long without someone noticing the diet-sex ratio link. This could be explained by the extremely generalist nature of feeding in *T. californicus* (Lewis *et al.* 1997), combined with the ability of many harpacticoid copepods to facultatively synthesize some fatty acids *de novo* (Brett *et al.* 2009). For example, in Fig. 6 of the Algae experiment, the clutch size in the fish food-fish food treatment drops drastically between clutches 1 and 2, but slowly climbs to become similar to the algae-algae treatment clutch size. The difference between clutch 1 and 2 is due to the diet of the mother, because the first clutch reflects the food she was eating in the wild, whereas the second clutch reflects her experimental diet. It is possible that the decrease in clutch size in the fish food-fish food treatment was due to the sudden diet change from one with EPA to one without, and the subsequent clutch size increase reflects the facultative ‘switching on’ of EPA synthesis machinery. No such drop or recovery is seen in the algae-algae treatment, perhaps because that diet always contained EPA. Conversely, it may be that *T. californicus* is capable of deriving all the EPA/DHA needed for reproduction *de novo*, in which case some other nutrient must be responsible for sex ratio variability. There is likely to be an interaction of age and diet effects on sex ratio, which I am unable to exclude, because my study did not specifically address female age.

This dependence of sex ratio and clutch size (or egg production/hatching success/larval survival) on diet (or fatty acids) might explain other reports regarding *Tigriopus* reproduction. For example, Takano (1971) found that *T. japonicus* eggs didn’t hatch after the 9th clutch, and attributed this to a lack of sperm; however, lack of sperm has not been reported in *T. californicus* (Burton 1985). Takano (1971) fed his copepods only cereal and soy flour, foods which undoubtedly were lacking in DHA/EPA (which is are marine-based; Brett *et al*. 2009). Improved population health during algae blooms in the lab (Burton and Feldman 1981; pers. obs.) could be the result of increased variety of algae and corresponding fatty acid profiles. The slight seasonal sex ratio variation in wild populations of *T. californicus* (Bateman and Anholt 2017) may be due to the prevalence of different types of algae (and their corresponding fatty acid profiles) in blooms throughout the season, rather than to temperature directly.

Fatty Acids Reflect the Environment

Fatty acids are ideal candidates for nutrients that must reflect environmental variability and influence copepod fitness, which is necessary, when considering how selection for environmental influence on sex determination might manifest in a polygenic system. DHA distribution in T-ISO increases slightly in high light conditions and decreases under low nitrogen levels (Sukenik and Wahnon 1991), indicative of the tight link between fatty acids and environmental factors. Fatty acid metabolism is linked to nitrogen and phosphorus levels in the environment; for example N- and P- limited diatom *Thalassiosira weissflogii* had reduced proportion and content of long-chain PUFAs (like EPA/DHA), and the dominant sterol (=hormone precursor, usually made from EPA) was halved in nutrient limited conditions (Klein Breteler *et al.* 2005). Not only that, but this effect carried through to copepod fitness: young copepodite stages of *Temora longicornis* and *Pseudocalanus elongatus* developed at significantly reduced rates when eating nutrient-limited diatoms, and at high levels of nutrient limitation, the copepods often did not reach maturity (Klein Breteler *et al.* 2005).

Tigriopus californicus PSD as a Model System

Bull (1983) recognized that his GSD/ESD framework of sex determination was limited, since he explicitly did not define it to encompass the zygotic perspective or to include an ontogenetic view of the sex determination process; the most common inconsistencies with this framework have been the steady reports of environmental influence on sex where none were expected (*e.g*. Rosenfeld and Roberts 2004). Now that researchers are more aware of the importance of parent-offspring conflict and environmental effects, several have been advocating for a re-evaluation of sex determination theory (Uller and Helantera 2011; van Doorn 2014; Perrin 2016). The *T. californicus* polygenic system is a useful and convenient model system in which to reconsider the relationships between sex determination and sex allocation.

There is evidence of environmental influence on sex even in systems with chromosomal sex determination, and it seems to be linked to the different ‘costs’ of the sexes (*i.e.* the fitness functions of the sexes increase differentially with increasing resources). In a review of sex allocation in mammals, Rosenfeld and Roberts (2004) state that classical Trivers and Willard-style condition dependence reduces to maternal diet and the age of the mother rather than the maternal body condition *per se*, and this is what influences sex ratio; for example, in mice, a diet high in saturated fats but low in carbohydrate results in a male sex ratio bias, whereas when the same number of calories are given as carbohydrate rather than fat, sex ratio is female-biased (males are considered more ‘expensive’ and are associated with more lipids).

Authors that have proposed re-evaluating sex determination and sex allocation include Uller and Helantera (2011), who thoroughly state their case based on the necessity of viewing sex determination as an ontogenetic process (including sex development) rather than as a ‘trigger’ (as in Bull 1983). According to van Doorn (2014), current theory largely models population-genetic aspects of sex determination, without accounting for individual-level selection on developmental mechanisms involved in sex determination; this is linked to the under-representation of zygotic selection on sex ratio in sex allocation. Perrin (2016) highlights the importance of stochastic noise in sex determination, and advocates for a three-ends continuum of sex determination (GSD, ESD, and ‘random’ sex determination); this paper was especially gratifying for me to read, because during my study of Bull (1983), I noticed that he used the same phrase ‘environmental effects’ to refer to both the predictable influence of an environmental variable on sex (in the context of ESD), and to the portion of the environmental influence that causes ‘random’ differences in development, such as that which determines left and right side symmetry (these stochastic ‘environmental effects’ in the context of PSD). I struggled with the realization that sex in a polygenic system seemed to be the sum of three, not two types of sex determining factors, and that I needed to reconcile this with the classical GSD/ESD and sex allocation frameworks. Indeed, much of Perrin's (2016) argument for a three-ends continuum rests on the idea that random stochasticity has an influence on sex determination comparable in size to traditional genetic and environmental sources, and his best example is polygenic sex determination.

In the *Tigriopus californicus* polygenic system, I began by looking for condition dependent sex allocation *sensu* Trivers and Willard (1973), expecting to find that maternal condition might influence her sex allocation decisions; while results do support a role for maternal diet in sex ratio variation, the effect of clutch diet (and therefore environmental sex determination) on sex ratio was more striking, and I argue that this is driven by fatty acids, specifically, EPA and/or DHA. I suggest that resource-dependent ESD and condition dependent sex allocation (after Trivers and Willard 1973) may therefore have a shared basis in fatty acid metabolism, though fatty acid composition is difficult to manipulate and measure in mammals, which would make tests of this hypothesis difficult to carry out.

Polygenic sex determination appears to be maintained as a consequence of specific relative fitness differences between genotypes, according to van Doorn (2014). A *Tigriopus californicus* polymorphism in the enzyme GPT is thought to be maintained by the patchy nature of environmental selection (Burton 1981), and a free amino acid polymorphism seems to be maintained by differential survivorship (Burton and Feldman 1983). Similarly, the sex polymorphisms (*i.e.* different combinations of sex-ratio linked alleles) of polygenic sex determination in *T. californicus* may be maintained by the patchy nature of an environmental parameter that acts selectively on the sexes. For example, of the ~6 loci identified in sex ratio variation in *T. californicus*, there might be one or more associated with fatty acid synthesis or metabolism, and sex differential selection on the ability to make or uptake fatty acids could correspond to the patchiness of fatty acid distribution in their habitat. Thinking of sex as a polymorphic trait rather than a binary one would help include systems with polygenic sex determination more readily in conceptual theory (Moore and Roberts 2013).

The *Tigriopus californicus* polygenic system sits at the crossroads of major themes in the evolution and maintenance of sex. Future research should include learning more about parent-offspring conflict and resource allocation in *T. californicus* and related copepod systems; studies of sex allocation in the harpacticoids (*e.g*. *Euterpina acutifrons*), cyclopoids (*e.g*. *Pachypygus gibber*) and calanoids (*e.g*. *Calanus* and *Temora* spp.) could provide some useful comparative information about the ancestral sex determining mechanism in copepod clades. The strength of environmentally based selection and parent-offspring conflict influences the genetic architecture of sex factors and the variability of sex determining systems in copepods.

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Appendix A: Pigment Analysis

I performed a pigment analysis to quantitatively ascertain whether *T. californicus* could uptake the supplementary synthetic β-carotene in the carotene diet treatment (from the Carotene Experiment). After I recorded clutch sex ratios in the Carotene Experiment, I kept males and females separate and froze them at -80°C (after starving them for 12-16h to empty their gut) so I could later quantify their levels of astaxanthin. I expected that copepods eating the algae and the supplemented carotene diet would show similar astaxanthin content, and that it should be higher than copepods fed a plain fish food diet (which was assumed to have few to no carotenoids, since copepods eventually turn white in the lab when eating it).

I made a standard curve to calculate copepod sample astaxanthin concentration based on known concentrations of lab grade astaxanthin (≥97% astaxanthin from *Haematococcus pluvialis,* Lot 125M4702V, Sigma-Aldrich Co.; Fig. A) in 90% acetone. The standard curve was run at 478nm, the absorption spectra peak for astaxanthin, as determined by literature and confirmed by running absorption spectra on a trial sample of pigment extracted from *T. californicus*. I based the range of concentrations for the standard curve on astaxanthin values reported in Goodwin and Srisukh (1949; ~5ug/copepod, *Tigriopus fulvus,* non-supplemented diet) and Caramujo *et al.* (2012; ~3ng/copepod, *Amphiascoides atopus*, diet supplemented with *H. pluvialis*).

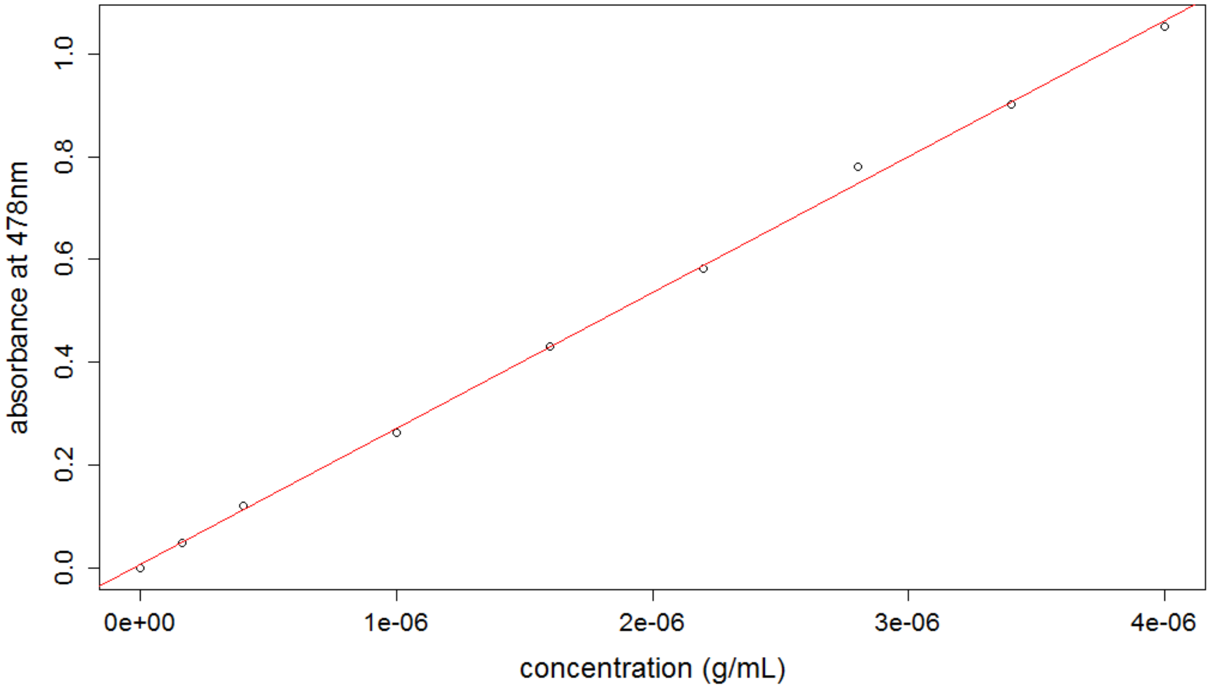

Figure A: Astaxanthin standard curve, read at 478nm, using >= 97% astaxanthin in 90% acetone. The diagonal represents the best fit line, equation: $y = 2.649 \times 10^{5} x + 6.011 \times 10^{-3}$, multiple $R^2 = 0.9988$, residual standard error =0.01444 on 7 DF, F-statistic =5753 on 1 and 7 DF.

Four samples each of males and females (n=200 copepods per sample) from each of three diet treatments (algae, carotene, and fishfood) were starved for >12h (to ensure their guts were empty) then homogenized in 90% acetone before being centrifuged and the supernatant (containing the pigment) separated. I read sample absorbance at 478nm using an Ultrospec 2100 Pro spectrophotometer, and analyzed results using ANOVA in R. Because I expected *a priori* that females and males should differ in the amount of astaxanthin present due to the inclusion of egg sacs in the female samples (as in Goodwin and Srisukh 1949), I analysed the sexes separately, and I report astaxanthin content in nanograms per copepod rather than per unit dry weight, due to limitations on resources and time (the drying process would likely affect the astaxanthin content in the sample and compromise the results without proper care). Values represent means ± standard deviation.

Females who were raised on the carotene- supplemented fish food contained significantly more mean (±SD) astaxanthin with 3.89 (±0.71) ng/copepod than females who ate either the plain fish food or the algae with 2.56 (±0.60) and 2.71 (±0.53) ng/copepod respectively (Table A, Fig. B). Males showed the same trend but the differences were not significant: the carotene diet showed the highest astaxanthin content with 3.53 (±1.27) ng/copepod, more than the fish food and algae diets with 2.73 (±0.80) and 3.08 (±0.99) ng/copepod (Table A, Fig. B).

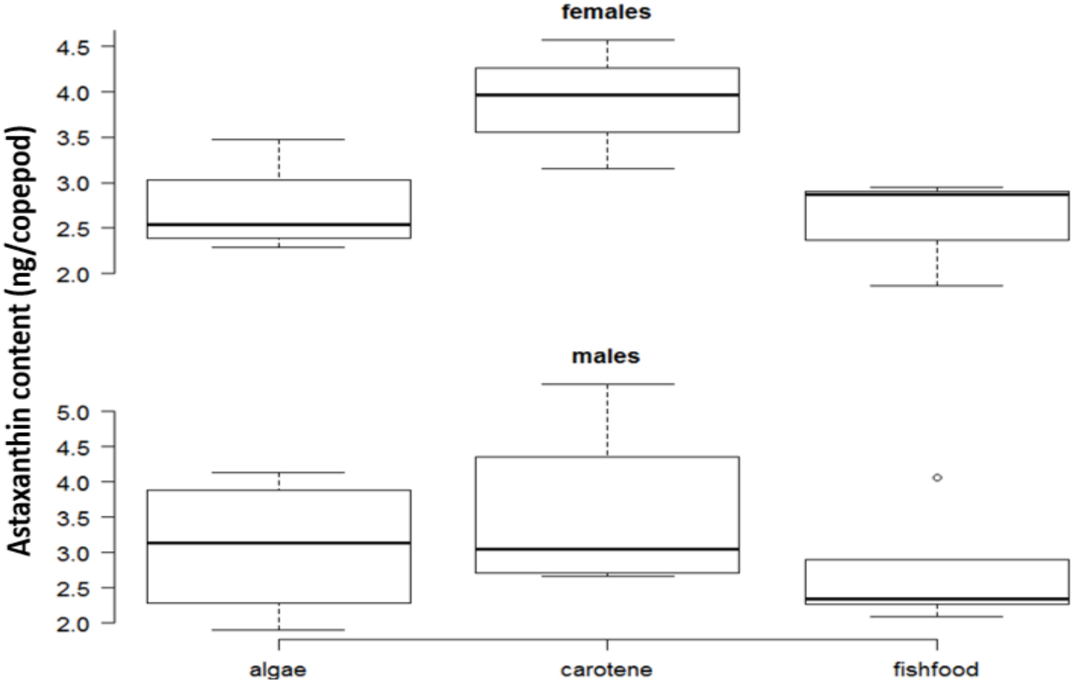

Figure B: Total body astaxanthin content from female and male copepods in three diet treatments: algae (left), carotene-supplemented fishfood (middle), and plain fish food (right), expressed in nanograms per copepod. Sample size was 4 for each diet/sex combination (except the carotene-fed females where one sample was lost); each sample consisted of 200 adult copepods. Box midlines represent medians, upper and lower box edges represent first and third quartiles.

These values compare to those reported by Caramujo *et al.* (2012) for the copepod *Amphiascoides atopus* when given diets of T-ISO paste supplemented with either

Haematococcus (3.10 ng/copepod) or *Spirulina* (~2.5 ng/copepod) meals. They found that the copepods were able to uptake and metabolize the extra carotenoids into astaxanthin evidenced by the large amount of astaxanthin in the *Spirulina*-supplemented copepods, since β-carotene is present in large amounts in *Spirulina*, with no astaxanthin, whereas *Haematococcus* has astaxanthin itself, not β-carotene. I conclude that *T. californicus* was able to uptake and metabolise the supplemented β-carotene.