Evolutionary Genetics Research
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Gaining insight in the sex determination mechanism of the parasitoid wasp *Nasonia vitripennis*

Introduction

Background

There are many different sex-determining mechanisms known in the animal and plant kingdom. In some species an environmental factor like temperature determines the sex, in others it is a gene or chromosome[1].

A special way of sex determination is present in the *Hymenoptera*, the animal group where bees, wasps and ants belong to. From haploid unfertilized eggs males arise, from diploid fertilized eggs females arise[2]. But haploid females[6] and diploid males[5] are also found in this species. A series of experiments also conclude that fertilization does not influence the sex.[5]

In bees the so-called *Complementary Sex Determiner* (*csd*) locus is found[9]. A great variaty of alleles are found on this locus. Heterozygotry on this locus results in the formation of a female individual, while hemizotry or homozygotry results in a male individual. In this way diploid males can arise if they carry only one type of allele. In other *Hymenoptera* species like some types of wasp, the *csd* is not found. These species have an other system for determining the sex, since inbreeding does not change the number of diploid males[4]. The commonly as an model organism used parasitoid wasp *Nasonia vitripennis* is one of the wasps where the sex determination mechanism is not fully understood. Because this wasp is used often in the laboratory as an model organism, it is important to learn more about it.

Model organism

Nasonia vitripennis is a small parasitoid wasp that lays its eggs in pupae of *Calliphora* flies[2]. It has a relatively short generation time and produces many offspring. Female wasps can lay both fertilized and unfertilized eggs.

To gain more insight in the sex determination mechanism of *Nasonia vitripennis,* mutant strains of this organism are used. A sex determination mutant strain is found in a wild population in Canada (CD_{12}). This strain produces so called gynandromorphs. Gynandromorphs are uniparental 'male' individuals with an anterior/posterior gradient of female external characteristics. The body parts that show these sexual dimorphic exterior are the antennae, the wings, the legs and the abdomen. If a body part is feminine, all anterior body parts are also feminine. This mutant strain is cultivated in the laboratory and directionally selected for more gynandromorphs. The allele that is probably partly responsible for the gynandromorphism in this strain is called *gyn1*.[5]

The other mutant strain that is used is the polyploid strain. This strain consists of diploid males and triploid females. Diploid males are sons of triploid females, and triploid females arise when crossing a diploid male and a diploid female. This polyploid strain has two recessive eye color markers. The allele *oyster* (oy) codes for grey colored eyes and the allele *scarlet* (st) for red colored eyes. The alleles for these eye colors are recessive and do not recombine.[6]

Application of the model organism

Several hypotheses are created to explain the sex determination mechanism in *Nasonia vitripennis.* The two main ones are the Maternal Effect Sex Determination (MESD) and the Genomic Imprinting Sex Determination (GISD).

MESD hypothesizes that the mother puts a gene product in her eggs during oogenesis which masculinizes her offspring. The offspring produce an other gene product, which counter-acts the masculinization. Offspring with n gene copies, will produce less of this gene product, and therefore the masculinizing effect of the mother is stronger than the demasculinizing effect, resulting in male offspring. Offspring with 2n gene copies will produce more demasculinizing products, therefore they will become female. Diploid sons are born out of triploid females, therefore they will receive more of the maternal gene product because she has more copies of the masculizing gene product gene.

GISD hypothesizes that the genes that are transmitted from males and females to their offspring are differently imprinted[8]. A specific gene on the genome is being imprinted by males but not by females. A gene product will bind to this specific gene if it is not imprinted, resulting in female offspring. Therefore females will produce sons when there are no male genes contributed to her offspring.

To find verification or falsification for one of these hypotheses, we are doing a series of crossing experiments. We are focusing on the influences of the degree of ploidy along with the maternal effects on the sex determination in *Nasonia vitripennis.*

Therefore the gynandromorphic mutant strain is crossed with the polyploid strain to obtain females that are both genetically predisposed to give rise to gynandromorphs and that are triploid. The offspring thereof will then be scored for the degree of gynandromorphism. This will be done at two different temperatures, because previous research has concluded that the rearing temperature is an important factor in the degree of gynandromorphism.

Offspring of virgin mothers with one, two or no *gyn1* alleles are scored for gynandromorphism. The frequency and degree of gynandromorphism in the progeny should conclude if either MESD or GISD is closer to the truth.

Research questions

The main question is how the sex of *Hymenoptera* is determined. To gain a little more insight the following research questions are proposed:

- How does the amount of allele *gyn1* in the mother influence the gynandromorphism of her offspring?
- How does the parental origin of the cytoplasm influence the level of gynandromorphism?
- What is the influence of the level of ploidy of the mother to the gynandromorphism of her offspring?
- How does the rearing temperature affect the degree of gynandromorphism?
- How is the ploidy body pattern of gynandromorphic individuals?[10]
- Does the ploidy of an individual influence the level of its gynandromorphism?

Hypotheses

The more of allele *gyn1* present in the mother, the more gynandromorphism in her offspring.
The parental origin of the cytoplasm is of no significant relevance.
The level of ploidy of the mother does not influence the level of gynandromorphism of her offspring.
The higher the rearing temperature, the more gynandromorphism.

The body of a gynandromorphic wasp has no pattern of cells with a deviating ploidy. The ploidy of an individual does not influence the level of its gynandromorphism.

Material and Methods

***Nasonia vitripennis* biology:**
Nasonia vitripennis is a cosmopolitan species of small parasitoid wasps. They measure up to 2 to 3 mm in length and usually do not fly. In the laboratory they are easily cultured on *Calliphora vicina* pupae hosts.[5]
Sex determination in *Nasonia* follows the haplodiploid system: from haploid (unfertilized) eggs males arise and from diploid (fertilized) eggs females arise.[5] Males and females are easily distinguished by their morphology. Females have black antennae, black bases of their legs, long wings and an ovipository tube inside their abdomen. Males have yellow antennae, yellow legs, short wings and their abdomen is pointier. Virgin females are obtained by opening the host pupae before the wasps emerge, and then separating the males and females on basis of their morphology when they are still in their pupal stage.[5]
Standard laboratory strains of *N. vitripennis* usually take 15 days to develop from egg to adult when cultured at 25˚C. Adult *N. vitripennis* can be kept alive for several months when keeping them at 4˚C.[5]

Strains:
The strains that are used are cultured in the laboratory after being caught in the wild. The gynandromorphic strain CD_{12} was caught in Canada and directionally selected in the laboratory for more gynandromorphs.[6]
The polyploid strain was sired by one male that accidentally was found to be diploid. This diploid male fertilized females whereby triploid daughters arose. These triploid females gave in their turn rise to haploid and diploid males. This polyploid strain has two recessive eye color markers. The allele *oyster* (oy) codes for grey colored eyes and the allele *scarlet* (st) for red colored eyes. The alleles for these eye colors are recessive and do not recombine.[6]

Definition of gynandromorphism
Gynandromorphic individuals from the CD_{12} strain are individuals that develop from unfertilized eggs, but instead of having only male external characteristics they show some female features. The wasps show an anterior/posterior gradient of female characteristics. The body parts that show these sexual dimorphic exterior are the antennae, the wings, the legs and the abdomen. If a body part is feminine, all anterior body parts are also feminine. Most gynandromorphs are symmetrical in their external characteristics, but some have only one feminine antenna or one feminine wing (along with two feminine antennae).

Scoring and pattern of gynandromorphism:
The frequency of gynandromorphs is defined as the percentage of gynandromorphs in the offspring of a female. Frequency of gynandromorphs = number of gynandromorphs / number of total offspring.
The degree of gynandromorphism is calculated by scoring the number of external characteristics that are feminine in gynandromorphs. The body parts that are scored include the antennae, the wings, the legs and the abdomen. For every female characteristic body part a gynandromorph possesses one point is added. One black antennae or one long wing counts as half a point. For example, a gynandromorph with feminine antennae and

wings receives 2 points. A gynandromorph that has a completely female appearance thus receives 4 points. A gynandromorph can so be scored for its degree of gynandromorphism. The offspring of a female can then be categorized for both the number of gynandromorphs and the degree of gynandromorphs. For calculating the total gynandromorph score of the offspring of a female, a male son counts as zero points, so is taking into account when the mean is calculated. Total gynandromorph score = total points of offspring / total number of offspring.
The wasps are put into the freezer for one hour to three hours before scoring. They are analyzed for the gynandromorphic characteristics under the binocular and their gynandromorph score is written down.

Temperature effects:
The temperature plays an important role in the development of gynandromorphs. Previous research shows that higher rearing temperature results in more gynandromorphs[5]. There seems to be a critical time for exposing the developing eggs to the high temperature. This time lies somewhere before oviposition by the female to about 8 hours after oviposition[5]. All our crossing experiments are conducted in duplo, one at 25˚C and one at 31˚C. The wasps are kept at this temperature shortly before or soon after emerging from their pupal stage, and staying there their entire lives.

Crossing experiments
Five crossing experiments have been preformed to test different effects on sex determination (Fig.1)
For the control group (A), the progeny of a diploid female from the CD_{12} strain is being scored for gynandromorphism. In theory, all the offspring will be haploids from unfertilized eggs.
The cross between a diploid female from the CD_{12} strain and a diploid male from the polyploid strain (B), results in triploid females and haploid males. The triploid females will have one (maternal) gynandromorph allele. These virgin triploid females will give rise to diploid and haploid individuals. These individuals will be scored for gynandromorphism.
The third experiment (C) is a cross between diploid females from the CD_{12} strain and diploid males that presumably carry one gynandromorph allele. They will also give rise to triploid females, now with two gynandromorph alleles. The progeny of the virgin triploid F1 females will be haploid or diploid and in turn will carry none, one or two gynandromorph alleles. These individuals will be scored for gynandromorphism.
The same will be done in the fourth crossing (D), but this time, the diploid male assumably carries two gynandromoph alleles, and therefore, the triploid daughters will carry three gynandromorph alleles.
Finally, a reciprocal cross (E) is done to test maternal effects on gynandromorphism. A triploid female from the polyploid strain is crossed with a haploid male from the gynandromorph strain. The offspring thereof are triploid and diploid females and haploid and diploid males. These virgin triploid females give rise to haploid and diploid progeny and the diploid females will give rise to haploid progeny. The progeny will be scored for gynandromorphism.

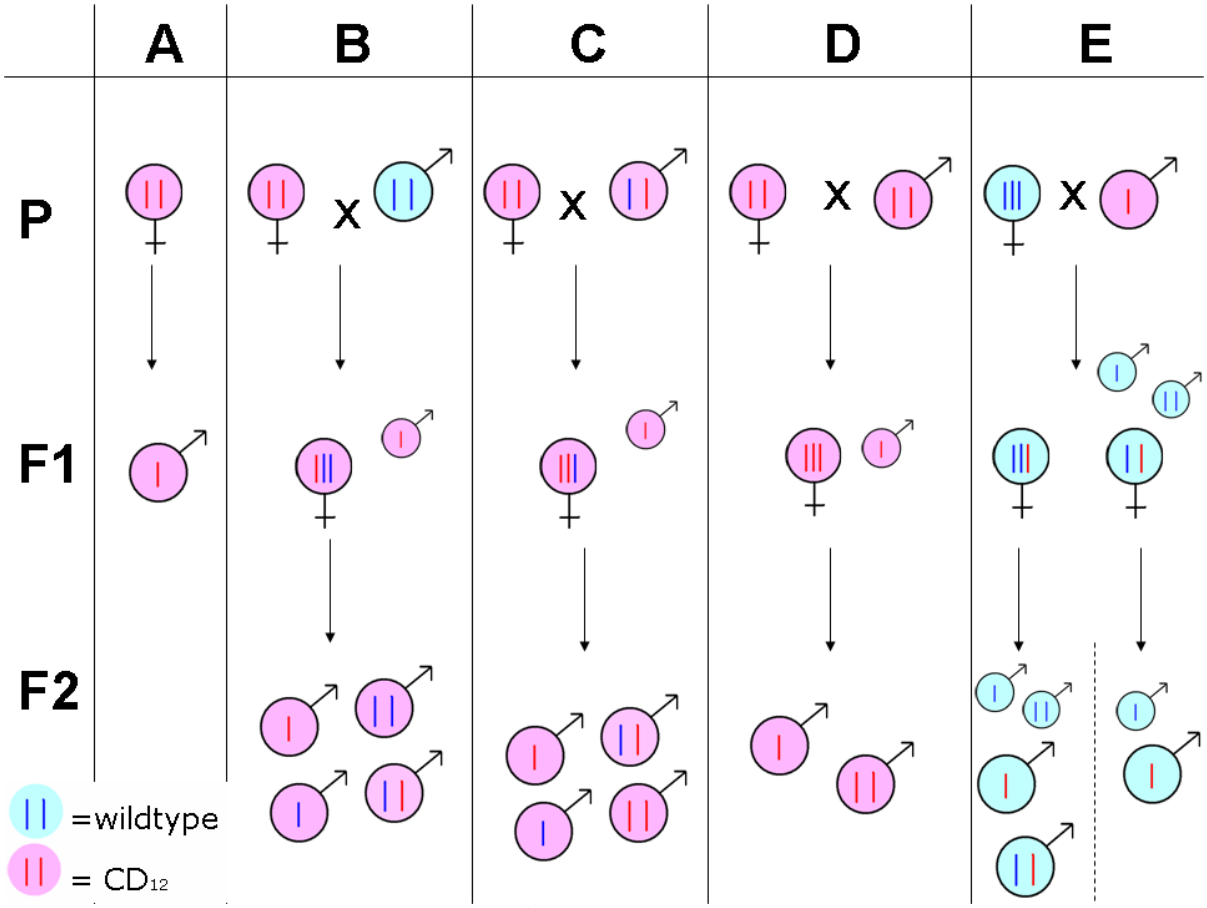

Figure 1. Crossing scheme of crossings A to E. ♂ are male indivuals, ♀are female individuals. The chromosomes and cytoplasm of individuals from the polyploid strain are marked in blue, the chromosomes and cytoplasm of individuals from the CD_{12} strain are marked in red . Individuals with one line are haploid, with two are diploid, and with three are triploid.

Measuring ploidy

To determine the ploidy of the F2 progeny, crossings are written down and the theoretical ploidy level is calculated. To be sure, a series of tests are conducted.

Flow-cytometry: Randomly chosen individuals are measured for ploidy using Flow-Cytometry. The head of a wasp is grinded using a glass grinder and suspended in 50 µl Galbraith buffer. The suspension is poured through a 50 µm diameter cell-strainer cap. 25 µl 1 mg/ml propydium iodide solution is added to colorize the DNA. The suspension is stored overnight or for 2 hours at 4˚C.

The samples are loaded into a Blackman Coulter Flow Cytometer EPICS XL-MCL and ran through until either 2500 cells are measured or 300 seconds have passed.

Individuals with recessive eye color markers are designated as controls for being either haploid or diploid. The peaks in the graphs for every ploidy degree are marked using a protocol. The mean number of cells in these marked areas is calculated and noted.

Allozyme electrophoresis: : The gynandromorphic strain and the polyploidy strain show a dimorphism in a specific protein. When these two protein morphs are ran through an electrophoresis gel, one of these morphs flows faster than the other. Therefore they are called *slow* and *fast.* The gynandromorphic strain is homozygotic for the fast morph, while the polyploidy strain is homozygotic for the slow morph. After grinding the wasps they are loaded into an electrophoresis gel for 45 minutes. A specific coloration is added to make the protein bands visible.

When two bands appear on the gel, the individual must be diploid. When a fast protein band is found, the individual must be haploid. Theoretically, half of the wasps that show a slow protein band are haploid and half are diploid (Fig. 1).
Number of progeny: Triploid *N. vitripennis* produce many aneuploid eggs, because of their meiosis. These eggs do not develop. Accidentally an euploid egg is produced, which does develop into an individual. Therefore the total number of offspring of triploid females is many times lower than of diploid females, which have no trouble in meiosis. Triploid females usually produce 2 or 3 % of the normal number of progeny. When the progeny of one female is counted, the ploidy of this female can be deduced.

Statistical analysis
To analyze the data statistically, a Chi^2-test is preformed as well as a T-test. Some of the data is transformed using the arcsin transformation.

Influence of temperature on gynandromorphism
Recent studies[5] have shown that the level of gynandromorphism is influenced by rearing temperature. To test this, each group (A to E) has been reared by 25 and 31 degrees Celsius. Because of ill-development of groups B, C, and D at 25ºC, and because of absence of gynandromorphism (as will be described later), the temperature effect can only be tested on the control group A.
There was no significant difference (T-Test of arcsin transformations, P=0.386) between the two temperature groups, when all gynandromorphs are grouped together. If a gynandromorph trait (i.e. black antennae, long wings, dark legs and feminine abdomen) is given a score, according to the scoring system, there was still no significant difference between the two groups (T-Test, P=0.203). This can be caused by the enormous variation between individuals.
Table 1. The mean of the groups is indicated as a proportion. The superscript letters indicate a significant difference between the groups. The same letter means no significant difference, a different letter means a significant difference using α=0,05. All groups were developed at 31°C.

Group	Proportion of gynandromorphs and uniparental ♀ among progeny	Gynandromorph score	Arcsin transformation of the proportions
A	0,28[a]	0,78[a]	31,49[a]
B	0,27[a]	0,73[a]	19,02[a]
C	0,71[b]	2,59[b]	61,40[b]
D	0,63[b]	2,17[b]	57,36[b]
E	0[c]	0[c]	0[c]

Ploidy body pattern
Analyzing the heads of gynandromorph individuals from unfertilized eggs from group A with flow-cytometry clearly showed haploidy. The abdomen of the same individuals however, did not give conclusive results. Therefore, nothing can be predicted about the ploidy body pattern and its association with gynandromorphism.

Ploidy of the gynandromorph
The ploidy of gynandromorphs and males from group B was analyzed using allozyme electrophoresis. When two bands appear on the gel, the wasp was diploid. When a fast protein band is found, the wasp was haploid. Theoretically, half of the wasps that show a *slow* protein band are haploid and half are diploid.

All diploid wasps are female looking gynandromorphs (Fig. 2). All female wasps show either two bands or the *slow* band.

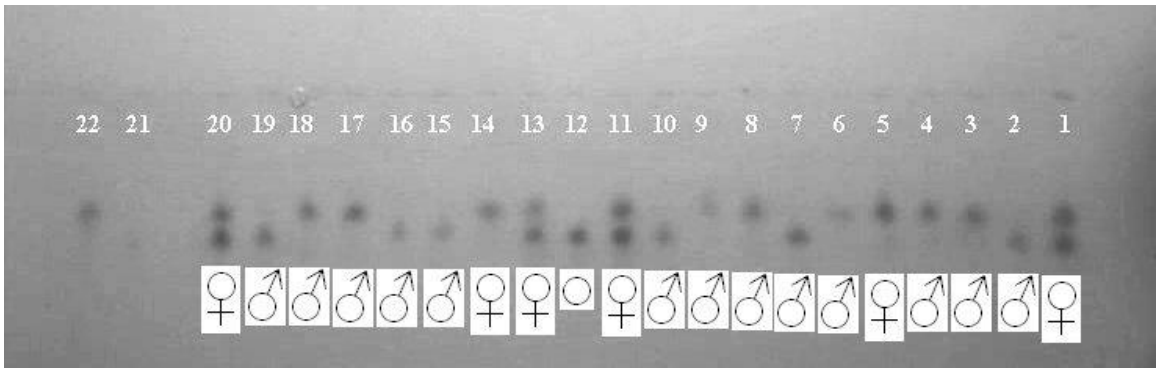

Figure 2. Allozyme gel electrophoresis of males and gynandromorphs of the B group. Two protein morphs are present in these strains. When run through an electrophoresis gel, one of these morphs flows faster than the other[11]. The black bands represent these proteins. The ♂ indicates an individual with complete masculine external characteristics, the ♀ indicates an individual with complete feminine external characteristics. Wasp number 12 was a gynandromorph with black antennae and long wings.

Importance of occurrence of gynandromorph allele

The ratio of occurrence of the gynandromorph allele in the parental genome could be quite important for the expression of gynandromorphism in the offspring. To test this, the level of gynandromorphism in the F2 of groups B, C and D were compared, where the maternal genome (F1) contained one, two or three gynandromorph alleles respectively. It appeared that a difference in gynandromorph expression existed between the F2 of groups B (one *gyn1* allele) and C (two *gyn1* alleles) (T-tests for the gynandromorph scores, and for the arcsin transformations of gynandromorph proportions: P<0,000), and between the F2 of groups B and D (three *gyn1* alleles)(T-tests for the gynandromorph scores, and for the arcsin transformations of gynandromorph proportions: P<0,000). There was no significant difference between the F2 of groups C and D (T-tests for the gynandromorph scores: P=0,289; T-test for the arcsin transformations of gynandromorph proportions: P=0,631) (Table1.).

Influence of cytoplasmic origin on gynandromorphism

Not only the nucleair, but also cytoplasmic DNA can influence the expression of certain traits, like gynandromorphism. To test if the maternal cytoplasm (F1) influences the gynandromorph expression of the offspring (F2), the F2 of groups B and E are compared. The F2 of these two groups have the same nuclear DNA, but differ in cytoplasmic origin. Individuals from group B have cytoplasm of the CD_{12} strain, where individuals from group E have cytoplasm of the polyploid strain.

The results quite clearly showed that there were no gynandromorph individuals in the F2 of group E, where there were gynandromorph individuals found in the F2 of group B. However, when tested, there was almost no significant difference found between the two groups (T-tests for the gynandromorph scores: P=0,060; T-test for the arcsin transformations of gynandromorph proportions: P=0,038) (Table 1.) because of the small sample size. This makes predictions about the effect of the origin of cytoplasm on gynandromorphism uncertain.

Influence of ploidy level on gynandromorphism

To test whether the maternal ploidy level influences gynandromorphism in the offspring, the F2 of group A and D have been compared. The mothers (P) of group A where diploid females from the CD_{12} strain, the mothers (F1) of group B where triploid females with

three gynandromorph alleles. The influence of maternal ploidy level can then be tested by comparing the haploid offspring of these mothers. However, the mothers of group D also provided diploid offspring, and in this research, no distinction has been made between the haploid and diploid progeny. For that reason, the difference that was found between the two groups (T-tests for the gynandromorph scores: P<0,000; T-test for the arcsin transformations of gynandromorph proportions: P=0,002) (Table 1.) does not have to be caused by differences in maternal ploidy level.

Conclusion and Discussion

After doing the experiments and collecting the results, a look can be cast on the research questions.

How does the amount of allele gyn1 in the mother influence the gynandromorphism of her offspring? When group B, C and D are compared, a significant difference is found between group B and C and between group B and D (Table 1.). Thus, the higher the amount of *gyn1* alleles and the higher the ratio *gyn1* to the 'normal' allele, the more gynandromorphism.

For more resolving power a larger sample size is neccesary. Especially group B had too few individuals to properly measure their mean number of gynandromorphs.

How does the parental origin of the cytoplasm influence the level of gynandromorphism? Group B posseses the cytoplasm of the gynandromorphic strain CD_{12} whereas group E posseses the cytoplasm of the polyploid strain. When scoring the gynandromorphism in these groups, group B had 27% gynandromorphs and uniparental females among the progeny whereas group E had none (Table 1). A very small significance was found when testing these data. So cytoplasm does influence the level of gynandromorphism.

A larger sample size would improve the resolving power of the experiment. The expectation is that a larger sample size will make the difference betwee group B and E highly significant.

What is the influence of the level of ploidy of the mother to the gynandromorphism of her offspring? When comparing group B, C and D with respectively one, two or three gyn1 alleles, a significant difference is found between group B and C and between group B and D (Table 1). This implies that the proportion of the gynandromorph allele in the maternal genome does influence the level of gynandromorphism in the offspring.

The sample size was too small to find a significant difference between groups C and D. A larger sample size would probably find a significant difference between these groups.

How does the rearing temperature affect the degree of gynandromorphism? When comparing the level and the degree of gynandromorphism of group A reared at 25ºC and at 31ºC, more gynandromorphs where found at 31ºC. However, this difference is not significant.

A larger sample size would probably make the differnce more significant. Groups B, C, D and E would also be compared when reared at 25ºC and at 31ºC, but because of the slower development of the groups at 25ºC there was not enough time to analyze the groups at 25ºC. Finding a significant difference between the temperature groups is expected.

How is the ploidy body pattern of gynandromorphic individuals? When analyzing the head and the abdomen of gynandromorphs and controls using Flow-Cytometry, no results could be obtained from the abdomen. Therefore, nothing can be concluded about the ploidy body pattern of gynandromorphs.

The heads of the gynandromorphs show clear haploidy. Because the theory predicts the feminine body parts to be diploid, one could take this as an indication that gynandromorphs do not have a ploidy body pattern.
To try to gain results from the abdomen using Flow-Cytometry, the digestive tract could be removed before preparing the sample. Other means of determining the ploidy of the wasps can be used, for example gel electrophoresis or quantitive PCR.
Does the ploidy of an individual influence the level of its gynandromorphism? When analyzing the allozyme electophorese gel (Fig. 2), all female loooking gynandromorphs are found to be diploid or have the *slow* band. Because all of the other diploids are female looking gynandromorphs, one could suggest that the females with the *slow* band are also diploid (theoretically half of the *slow* bands belong to diploid individuals). This cannot be concluded without further research and a larger sample size.
To gain further results, more wasps should be analyzed using allozyme electrophoresis. All degrees of gynandromorphism should also be analyzed. It would be great to find a way to distigious the haploid and the diploid *slow* band.

The sex determing mechanism of *Nasonia vitripennis* remains unclear, but a few steps forward have been made to learn more about the details.

The MESD model for sex determination is may be falcified, because the prediction that diploid sons are born out of triploid females because triploid females produce more of a masculinizing gene product is not true (Fig. 2). But this could be caused by the *gyn1* allele, that could be resonsible for a variant of the masculinizing gene product that does not function accurately.

Further research is needed to determine which of the sex determining models is closer to the truth and to determine further details of the sex determining mechanims of *Nasonia vitripennis.*

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