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Effects of Flower Color on Pollination and Seed Production in Lupinus Perennis

Honors Project

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Abstract:

We examined how flower color morphs (blue vs white) in *Lupinus perennis* affect the probability of setting fruit, average mass of a seed produced, and average number of seeds per pod. Samples were collected at Wintergarden Park, Bowling Green, Ohio, with a total of 10 blue flowered families and 7 white flowered families. A total of 426 seeds were catalogued and weighed. Individual stalks were scored for developed flowers, undeveloped flowers, as well as developed and undeveloped pods. Values for each stalk were averaged across maternal families. We found that blue flowered plants had a higher probability of setting fruit, possibly relating to a higher pollination rate. Blue flowered plants also produced seeds that had on average a higher mass. There was not a significant difference between the number of seeds per pod between the two flower color morphs.

Introduction:

The variety of flower structures in nature is incredible. Even amongst individual species, there is very visible variety. One such form of variation is color polymorphism, which is very common in plants. Color polymorphism refers to a single interbreeding population with two or more distinct color morphs. In true color polymorphism, the rarest color morph is too frequent to be solely the result of recurring mutations (Gray & Mckinnon, 2007). Essentially one species comes in a variety of colors. One morph is often at an advantage, and yet the disadvantaged color morph persists and is not wiped out from the population.

In some cases, this can be explained by frequency dependent selection. Often in natural selection, one trait seems to be objectively better for a given environment. A mouse with dark colored fur is going to fare better on dark soil than a white colored mouse on the same soil, because it will more successfully hide from predators. Therefore, darker fur is more advantageous, and will continue to be so as long as the background remains dark. With frequency dependent selection, neither color morph is objectively more advantageous. Instead, advantage is conferred in relation to the frequency of the trait in the population and the levels of environmental variation. With positive frequency dependent selection, the more common color morph is more fit. With negative frequency dependent selection, the rarer color morph is more fit (Gigord, Macnair & Smithson, 2001).

Pollinators can contribute to frequency dependent selection on flower color, by favoring a certain color morph when it comes to their foraging habits. In some cases, bees prefer the more common morph (Smithson & Macnair, 1996). In other cases, bees prefer the rare morph (Gigord, Macnair & Smithson, 2001). Another study suggests that bees as a whole do not have a preference, but instead individual bees will prefer one morph, with different bees from the same hive having different preferences (Jones & Reithel, 2001).

In cases with positive frequency dependence, the rare morph is discriminated against. This could result in a higher rate of self pollination in the rare morph than in the common morph. This allows the rare color morph to continue to persist in the population (Jones & Reithel, 2001), at a relatively stable ratio to the common morph. If there was instead negative frequency dependent selection, the rare morph would be favored by pollinators. This would result in plants with the rare morph producing more offspring, gradually shifting the frequency until the formerly rare morph is now more common. This would then reverse the trend, and we would see shifts back and forth over which flower color morph is more common.

However, color polymorphism is not always the only trait in play. In some plants, flower color changes over time. In many cases, this color change corresponds with rewards being offered to pollinators in the form of pollen and nectar (Kudo, Ishii, Hirabayashi & Ida, 2007). If a flower stops producing rewards when it is pollinated, then the color change links to reward levels associated with pollination status of the flower. This then raises the question, why go to all the effort of maintaining pollinated flowers, and changing the flower color? What is the benefit? The benefit is in how pollinators relate to the flower displays. From a distance, the factor that determines if a bee will visit is the size of the flower display. Larger floral displays attract more bees, regardless of the color status. Once bees get closer to the plant, they then take into consideration which individual flowers indicate rewards. Therefore, retaining older flowers with a color change increases the chance of unpollinated flowers becoming pollinated. Losing flowers once they are pollinated would result in a smaller display and the odds of the other flowers in the display being pollinated are low. If they retain older flowers with no color change, then bees will not know which to visit, and the flowers will not be pollinated as effectively (Kudo, Ishii, Hirabayashi & Ida, 2007).

My project examines the consequences of flower color variations in a plant of conservation significance, *Lupinus perennis*, also called Wild Lupine or Sundial Lupine,

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and hereafter referred to as simply *L. perennis*. It is a wildflower native to Ohio, and much of Eastern North America (Michaels, Mitchell & Shi, 2005).

L. perennis is a perennial that consists of a rosette growth form, with an average of 6-7 inflorescences, each with 50-60 individual flowers present (Michaels, Shi & Mitchell, 2007). It grows in nutrient poor areas, with sandy, well drained soil. It is seen as an indicator of quality oak savanna habitat, which is a mosaic of black oak savanna, wet prairie communities and oak woodland. It is the only host plant for the federally endangered Karner blue butterfly (Michaels et al., 2005). It is also a significant food source for other Ohio butterflies: the Persius Dusky Wing and the Frosted Elfin (Grundel & Pavlovic, 2009).

L. perennis is interesting due to its coloration, which varies during flowering. Flowers begin as a light blue, and darken as time goes on, beginning at the bottom of the inflorescence where the first flowers open and moving upwards. This change is thought to match up with pollination and reward for pollinators.

L. perennis also has color polymorphism. Most of the plants produce flowers that start light blue and transition to darker blue, but some begin white and transition to light blue. Currently, white flowered *L. perennis* are the rarer flower color morph. This would suggest that bees base their expectations on rewards on the more abundant blue flower color morph. This would mean associating light blue with rewards and dark blue with a lack of rewards. It is possible that white flowers would also be associated with rewards, as they are very light in color. Therefore, flowers that change from light blue to dark blue would only be attractive at their light blue stage. Flowers that start out white and then transition to light blue might be associated with rewards for the duration of their

bloom. Therefore, white flowered *L. perennis* plants would receive a rare morph advantage, due to pollinator preferences.

Hypothesis:

White flowered *L. perennis* experiences a negative frequency dependent selection because it is visited more by bees than blue flowered *L. perennis*. This would result in a higher probability of pollination for white flowers.

Methods:

I collected samples at Wintergarden Park, in Bowling Green, Ohio, during the summer of 2019. L. perennis is extremely plentiful there. I obtained permission to go off trail and collect plant material from park officials before proceeding. On May 31st, 2019 I manually surveyed fields for white flowered *L. perennis*. White flowered *L.* perennis is relatively rare in these fields, so I selected what plants I could find that were reasonably accessible. Most cases were a single white plant surrounded by blue plants for at least 5 m in all directions. When multiple white plants were near each other, I selected only one. I marked the location of 14 white flowered plants with flags, and recorded their rough location in a notebook. 14 blue flowered L. perennis plants were selected near labeled white flowered plants. These blue flowered plants were chosen as they were similar in size and development as the equivalent white flowered plant. For each plant, 2-4 stalks were selected and labelled with a piece of tape, indicating initial flower color. The tape was applied loosely below the flower structures so as not to impede growth or pollination. As many plants grew near each other, efforts were made to match leaf shape and other morphological traits to ensure that the stalks were from the same plant. Between two and three weeks later, when flowers had been

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pollinated and seed pods were developing, I returned to Wintergarden. I placed bags made from bridal veil material over the selected stalks, using twist ties to secure the bags closed. This is because the seed pods shatter upon maturity, sending their seeds flying. By July 10th, pods had fully matured and burst, and I returned and collected the stalks. Each set of stalks was placed in a paper bag labelled with the sample site and flower color. In cases where the mesh bags were damp from rain or dew, they were spread out and left overnight to dry before being placed in a paper bag. Stalks were stored in a dark box and allowed to dry. Some plants were marked but were not collected. These were cases where herbivores had grazed down the stalks, or some other event had happened to the plant that caused me to be unable to locate it again. This eliminated three white flowered plants and two blue flowered plants.

Each set of stalks is considered a family, with a "mother plant" and offspring in the form of seeds. Four white flowered families were eliminated on the basis of mold growing on the pods, none of the pods bursting, or seeds had germinated in the bag. Two blue flowered families were eliminated for the same reasons. This left me with eight blue flowered plants, and nine white flowered plants. However, upon further examination, three families had one stalk that produced drastically different colored seeds. I decided to categorize these stalks as being a different family. When I collected the stalks, I accidentally collected stalks from a neighboring plant as well, due to how closely the plants grow. This left me with 10 blue flowered families, and 7 white flowered families.

I created codes to distinguish families, and to convey relevant information, such as flower color. I then examined each stalk, labelling them with a letter code. For each stalk, the number of seeds was counted and recorded. The data was assembled so that I had the total number of seeds produced by each family, and from which stalk each seed came from. I gave each seed an ID code based on its family, and the stalk it came from. I weighed and recorded the mass of each seed to an accuracy of a tenth of a gram, using a Mettler Toledo Model AE-240 balance. A total of 426 seeds were individually weighed and assigned seed codes. Unusual appearances were also recorded. Each individual seed was placed in a centrifuge tube, labelled with its code, for storage, so they would not mix and become unidentifiable. These centrifuge tubes were stored in a refrigerator, at approximately 4.5° C.

To determine the number of flowers that had been on a stalk that produced the seeds, I then scored the stalks themselves. A pedicel connects flowers to the main stalk of the inflorescence. After the flowers have developed and withered away, the pedicel may remain on the stalk, or it may break off. Either way, it leaves behind a mark called a flower scar. These scars can be counted to determine how many individual flowers were on a single inflorescence. I counted and recorded the flower scars, dividing them into three categories, large, small, and pod breaks. Small scars had an average diameter of 0.5-0.75 mm, and large scars had an average diameter of 1- 1.5 mm. Pod breaks had an average diameter of 1.5-1.75 mm. Large scars were considered to be flowers that fully developed and opened, and therefore were available to be pollinated. Small scars were considered to be flowers that never matured, staying as buds. Pod breaks were scars that appeared to be a result of a flower being pollinated, beginning to produce a pod, and then breaking off. Pod breaks were distinguished by being larger than large flower scars, displaying more damage to the

stalk, and occasionally a large pedicel was still present. Pod breaks corresponded with pods found in the mesh bag, in all but one case. In one case, there was not a corresponding pod, which would indicate that the pod had broken off and fallen to the ground before I bagged the stalks. The pods that corresponded to pod breaks were at all stages of development, undeveloped, developed but intact, and developed and burst.

These flower scars were utilized to determine the total number of flowers, total number of developed flowers, and pollination rates. Total number of flowers was calculated by adding together the number of small scars, large scars, and total pods. Pods were included in the flower count as each pod would have developed from a single developed flower. Pod breaks did not contribute to the count as they corresponded with the number of pods. The number of developed flowers was calculated by adding large flower scars and total pods, once again not including pod breaks. Rates of pollination were calculated by dividing total pods by total developed flowers. Developed flowers were used instead of total flowers as the smaller, undeveloped flowers were not available for pollination.

In addition to flower scars, I recorded the total number of pods, burst pods, intact pods, and undeveloped pods. Intact pods were cracked open and their contents were recorded. In most cases, the seeds were tiny, and undeveloped. This would indicate that the ovules were fertilized, but were aborted for some reason. Some such pods had a white mold within them, covering the seeds. It is unclear if this mold infection caused the plant to abort the pod, or if the mold infection occurred after the pod had already been aborted. Other intact pods contained a mix of mature seeds and undeveloped seeds, and one pod contained only mature seeds. This final pod was likely not an abortion, but just simply a failure to burst. Fully developed pods were approximately 3-4 cm in length. Undeveloped pods were approximately 1-1.5 cm in length. Undeveloped pods were typically no longer attached to the stalk, and were found within the bag. This would indicate that either I had inadvertently knocked them off of the stalk during the bagging process, or that the plant had aborted them after they had been bagged. I took care to avoid knocking off pods, and do not recall doing so, so the abortion explanation is more likely. Some burst pods burst in such a way that a seed was trapped. The pods resemble pea pods when they first develop, with two halves that come together lengthwise, with the seeds nestled between them. They remain green as they develop and increase in size, with a white pubescent fuzz on their surface. As they mature, a dark stripe appears on the upper seam of the pods. This stripe expands over time until the entire pod is black and partially dried out. At this point, the two halves of the pod separate, sending seeds flying. The two halves are still somewhat flexible, and curl against themselves. Occasionally, seeds become trapped in the curls of the pods, which later dry out and become inflexible and brittle. These trapped seeds were initially missed when seeds were first counted and weighed. Mature seeds from intact pods as well as trapped seeds in burst pods were recorded as extra seeds. A total of 29 extra seeds were recorded. Stalk scoring and extra seed counting occurred during the online learning period away from BGSU's campus, so I was unable to weigh the extra seeds, as I lacked the proper equipment.

Data Analysis:

Data analysis was done in conjunction with Dr. Michaels, as she had greater access to resources for statistics such as JMP.

When comparing pods / developed flowers, I averaged the stalks for each family, to account for the variation in stalk numbers between maternal plants. We then used this data set of averaged stalks, and performed a T test assuming unequal variances in JMP.

We compared the average mass of a seed produced by each flower type. <u>First</u>, <u>we calculated the per family average across stalks of the</u> 268 seeds from 10 families of blue flowered plants. Similarly, 158 seeds from 7 families of white flowered plants were averaged across stalks per family. This data was analyzed using a nonparametric analysis using a Wilcoxon approximation in JMP software.

We examined the rate of seeds per pod, with a sample size of 10 blue flowered families and 7 white flowered families, also using the averages across stalks for each family. We performed a t-test assuming unequal variances, and a nonparametric analysis of the full data using a Wilcoxon approximation.

Results:

When I compared the number of pods / number of developed flowers to determine the probability of pollination between the two flower colors, I found that blue flowers had a greater probability of a flower developing a fruit (Figure 1). This is most likely a result of pollination, suggesting that bees visited blue flowers more often than white flowers. A T test assuming unequal variances resulted in a t Ratio = -2.388, DF =

14.66, Prob |t| = 0.0308, indicating the difference between means is significant. This analysis was done using the average fruits per flower per stalk for each family, to account for the variance in stalk numbers between maternal families <u>and to avoid</u> <u>pseudoreplication</u>. Ten blue flowered and seven white flowered families were used.



Figure 1. X axis depicts flower color. Y axis is probability of pollination, calculated as total pods / developed flowers.

When we compared the average mass of a seed from blue flowered plants to that of white flowered ones, blue flowered plants produced seeds that had on average a higher mass (average seed mass_{blue} = 26.62 mg vs average seed mass_{white} = 23.77 mg). We performed a nonparametric comparison of the means using a Wilcoxon 2sample test, normal approximation₇ which produced s= 44, |Z| = -1.80542, P= 0.0705, which is "weakly significant" (JMP v. 14). It indicates a trend that might be significant if the study were repeated with a larger sample size.

When we examined the rate of seeds per pod, analysis found that the data had a normal distribution (when one outlier was removed). 10 blue flowered families were used, and 7 white flowered families, also using the averages across stalks for each family. Both a t-test assuming unequal variances (t ratio = -0.583, DF = 7.17, p > |t| = 0.577) and a nonparametric analysis of the full data using a Wilcoxon approximation (s= 46, |Z| = -0.49, Prob>|Z| = 0.625), indicated that there was no real difference in seed/pod for the two flower morphologies. This is partially a result of the low number of ovules per flower in *L. perennis* seed pods. Most average at about six ovules per pod. There simply is not enough room to detect much variation. This might be possible with a very large sample size, taking into account different populations.

When we compared the number of flowers per stalk, analysis found no difference between blue and white flowered families. Mean flowers_{Blue} = 25.26 ; Mean flowers_{White} =

27.12. We performed a Wicoxon 2-sample test, with normal approximation (S = 69, Z = 0.537, Prob > |Z| = 0.59).

Discussion:

Despite the restrictions placed on my experiment by quarantine, I was able to observe a significant difference between the two flower color morphs. Blue flowered L. *perennis* have a higher probability of setting fruit than the white flowered. This contradicts my hypothesis, as I predicted that white flowered plants would have a higher probability of setting fruit. It is interesting that there is a difference, however. This may be a result of higher pollination rates. Higher pollination rates could be caused by pollinators favoring blue flowered plants over white flowered plants. I would suggest an observational study where bee habits are recorded specifically with L. perennis, to see if bees favor one flower color morph over the other. My initial thought was that flowers that transition from white to light blue would be more appealing to bees than flowers that transition from light blue to dark blue. However, it is possible that bees did not recognize white flowers as having a reward, and therefore did not visit them as frequently as they visited light blue flowers. This would result in white flowered plants self pollinating more than blue flowered plants. A previous study found that self pollinating in *L. perennis* leads to a lower ratio of fruit to flowers (Michaels, Mitchell & Shi, 2005), which matches up with the data observed in this study. We cannot completely conclude that the lower probability of flowers setting fruit is a result of decreased pollinator interest, but it is possible, and lines up with the data. Further study would be needed to confirm this.

Blue flowered plants also produced seeds that had a higher average mass than white flowered plants. The difference was only weakly significant, but I believe it would be significant if the experiment was repeated with a larger sample size. In fact, I strongly support this experiment being repeated. A larger sample size would allow for more accurate results. My sample size was limited to begin with, and further decreased by the time I actually collected the stalks. There were some stalks that were grazed by animals before I was able to collect them. Other stalks succumbed to mold. A larger sample size would mean such losses have less of an effect on the final result. It might be worth examining if either morph is more vulnerable to grazing from herbivores or infection by mold. In addition, it would be worth germinating the seeds to see if either morph produced fitter offspring. I'd be interested to see if the trend holds in other *L. perennis* populations, as then the information could be more easily used in efforts to reintroduce *L. perennis* and extend the range of the Karner blue butterfly.

	Family					Undeveloped	
Color	Code:	Stalk	Total seeds	Total pods	Open pods	pods	Intact pods
blue	1BN	A	24	8	7	0	1
blue	1BN	В	34	12	11	0	1
blue	1BN	С	23	8	8	0	0
blue	1BN	AVG	27	9.333333333	8.666666667	0	0.666666667
blue	18BN	A	23	8	8	0	0
blue	18BN	В	3	4	4	0	0
blue	18BN	С	12	3	3	0	0
blue	18BN	AVG	12.66666667	5	5	0	0
blue	3BNA	A	22	9	8	1	0
blue	3BNA	В	18	8	7	0	1
blue	3BNA	AVG	20	8.5	7.5	0.5	0.5
white	18WN	A	23	9	9	0	0
white	18WN	В	14	6	5	0	1
white	18WN	AVG	18.5	7.5	7	0	0.5
white	17WN1	A	14	4	4	0	0
white	17WN1	В	12	3	2	0	1
white	17WN1	С	11	5	4	0	0
white	17WN1	AVG	12.33333333	4	3.333333333	0	0.3333333333
white	5WS	A	8	5	2	3	0

white	5WS	В	18	7	7	0	0
white	5WS	AVG	13	6	4.5	1.5	0
blue	16BNA	A	8	4	4	0	0
blue	16BNA	В	18	7	7	0	0
blue	16BNA	AVG	13	5.5	5.5	0	0
white	15WN	A	5	2	2	0	0
white	15WN	В	8	3	3	0	0
white	15WN	С	16	4	4	0	0
white	15WN	AVG	9.666666667	3	3	0	0
blue	2BN	A	17	5	5	0	0
blue	2BN	В	6	3	3	0	0
blue	2BN	С	3	2	2	0	0
blue	2BN	D	0	9	0	9	0
blue	2BN	AVG	6.5	4.75	2.5	2.25	0
white	2WN	A	22	7	7	0	0
white	2WN	В	0	5	0	0	5
white	2WN	AVG	11	6	3.5	0	2.5
blue	12BNA	A	19	7	7	0	0
blue	3BNB	A	24	9	6	1	2
blue	12BNB	A	14	6	6	0	0
white	16WN	A	7	5	3	1	1
white	16WN	В	4	2	2	0	0

white	16WN	AVG	5.5	3.5	2.5	0.5	0.5
blue	16BNB	A	10	3	2	0	1
white	17WN2	A	7	2	1	1	0
blue	7BS	A	7	2	2	0	0

	Family						Developed
Color	Code:	Stalk	Small Flower	Large Flower	(pod breaks)	Total Flower	Flowers
blue	1BN	A	9	17	2	34	25
blue	1BN	В	12	15	12	39	27
blue	1BN	С	12	14	0	34	22
blue	1BN	AVG	11	15.33333333	4.666666667	35.66666667	24.66666667
blue	18BN	A	10	16	2	34	24
blue	18BN	В	17	14	0	35	18
blue	18BN	С	16	19	2	38	22
blue	18BN	AVG	14.333333333	16.33333333	1.3333333333	35.66666667	21.333333333
blue	3BNA	A	4	13	1	26	22
blue	3BNA	В	13	9	2	30	17
blue	3BNA	AVG	8.5	11	1.5	28	19.5
white	18WN	A	6	12	4	27	21

white	18WN	В	10	14	0	30	20
white	18WN	AVG	8	13	2	28.5	20.5
white	17WN1	A	22	19	1	45	23
white	17WN1	В	13	13	3	29	16
white	17WN1	С	11	14	0	30	19
white	17WN1	AVG	15.33333333	15.33333333	1.3333333333	34.66666667	19.33333333
white	5WS	A	0	13	5	18	18
white	5WS	В	6	18	7	31	25
white	5WS	AVG	3	15.5	6	24.5	21.5
blue	16BNA	A	8	13	0	25	17
blue	16BNA	В	19	13	3	39	20
blue	16BNA	AVG	13.5	13	1.5	32	18.5
white	15WN	A	8	26	0	36	28
white	15WN	В	14	14	0	31	17
white	15WN	С	13	19	0	36	23
white	15WN	AVG	11.66666667	19.66666667	0	34.33333333	22.66666667
blue	2BN	A	17	17	0	39	22
blue	2BN	В	17	0	0	20	3
blue	2BN	С	6	20	0	28	22
blue	2BN	D	8	5	9	22	14
blue	2BN	AVG	12	10.5	2.25	27.25	15.25
white	2WN	A	10	13	2	30	20

white	2WN	В	0	16	0	21	21
white	2WN	AVG	5	14.5	1	25.5	20.5
blue	12BNA	A	0	7	2	14	14
blue	3BNB	A	0	10	1	19	19
blue	12BNB	A	6	7	0	19	13
white	16WN	A	6	9	1	20	14
white	16WN	В	5	14	0	21	16
white	16WN	AVG	5.5	11.5	0.5	20.5	15
blue	16BNB	A	4	15	0	22	18
white	17WN2	A	4	17	3	23	19
blue	7BS	A	12	6	0	20	8

	Family		Avg Mass of		pods/ D.
Color	Code:	Stalk	1 seed	seed/D. pod	Flowers
blue	1BN	A		3	0.32
blue	1BN	В		2.833333333	0.44444444
blue	1BN	С		2.875	0.363636364
blue	1BN	AVG	0.024457143	2.892857143	0.378378378
blue	18BN	A		2.875	0.3333333333
blue	18BN	В		0.75	0.222222222

blue	18BN	С		4	0.136363636
blue	18BN	AVG	0.026681579	2.533333333	0.234375
blue	3BNA	A		2.75	0.409090909
blue	3BNA	В		2.25	0.470588235
blue	3BNA	AVG	0.028727027	2.5	0.435897436
white	18WN	A		2.555555556	0.428571429
white	18WN	В		2.3333333333	0.3
white	18WN	AVG	0.021643243	2.466666667	0.365853659
white	17WN1	A		3.5	0.173913043
white	17WN1	В		4	0.1875
white	17WN1	С		2.75	0.263157895
white	17WN1	AVG	0.021818182	3.363636364	0.206896552
white	5WS	A		4	0.277777778
white	5WS	В		2.571428571	0.28
white	5WS	AVG	0.031052	2.888888889	0.279069767
blue	16BNA	A		2	0.235294118
blue	16BNA	В		2.571428571	0.35
blue	16BNA	AVG	0.024353846	2.363636364	0.297297297
white	15WN	A		2.5	0.071428571
white	15WN	В		2.666666667	0.176470588
white	15WN	С		4	0.173913043
white	15WN	AVG	0.025436	3.222222222	0.132352941

blue	2BN	A		3.4	0.227272727
blue	2BN	В		2	1
blue	2BN	С		1.5	0.090909091
blue	2BN	D		#DIV/0!	0.642857143
blue	2BN	AVG	0.023491304	2.6	0.31147541
white	2WN	A		3.142857143	0.35
white	2WN	В		0	0.238095238
white	2WN	AVG	0.01916	1.833333333	0.292682927
blue	12BNA	A	0.033721053	2.714285714	0.5
blue	3BNB	A	0.027094444	3	0.473684211
blue	12BNB	A	0.0298	2.3333333333	0.461538462
white	16WN	A		1.75	0.357142857
white	16WN	В		2	0.125
white	16WN	AVG	0.0267	1.833333333	0.233333333
blue	16BNB	A	0.025911111	3.3333333333	0.166666667
white	17WN2	A	0.020842857	7	0.105263158
blue	7BS	A	0.031528571	3.5	0.25

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