Title: High mitochondrial mutation rates in *Silene* are associated with nuclear-mediated changes in mitochondrial physiology

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Abstract

Mitochondrial (mt) respiration depends on proteins encoded both by the mitochondrial and nuclear genomes. Variation in mt-DNA mutation rates exists across eukaryotes, although the functional consequences of elevated mt mutation rates in some lineages remain underexplored. In the angiosperm genus *Silene*, closely related, ecologically similar species have either 'fast' or 'slow' mt-DNA mutation rates. Here, we investigated the functional consequences of elevated mt-DNA mutation rates on mt respiration profiles of *Silene* mitochondria. We found that while overall levels of respiration were similar among species, fast species had lower respiration efficiency and relied up to 49% more than slow species on nuclear-encoded respiratory enzymes alternative oxidase (AOX) and accessory dehydrogenases (DHex), which participate in stress responses in plants. However, not all fast species showed these trends. Respiratory profiles of some enzymes were correlated, most notably AOX and DHex. We conclude that subtle differences in mt physiology among *Silene* lineages with dramatically different mt mutation rates may underly similar phenotypes at higher levels of biological organization, betraying the consequences of mt mutations.

1. Introduction

The mitochondria (mt) of most eukaryotes contain their own genome which encodes essential proteins that form the functional core of the mitochondrial electron transport system (ETS, figure 1a). The primary function of the ETS is to generate ATP via oxidative phosphorylation (OXPHOS) which provides a critical resource for myriad cellular functions. Proteins encoded by mt-DNA alone, however, are insufficient for OXPHOS. Many nuclear (N) DNA-encoded proteins are targeted to mitochondria and interact with mt encoded protein subunits to form functional chimeric ETS complexes (figure 1a, e.g., CI, CIII, CIV, and CV). The resulting mito-nuclear interactions are proposed to be critical for OXPHOS and other mitochondrial functions, and to play broad roles in evolution [1–3].

The ETS also contains strictly N encoded proteins that act as alternative entry (alternative NAD(P)H dehydrogenases; DHex) and exit (alternative oxidase; AOX) pathways for electrons (figure 1a) that are activated under certain cellular conditions to maintain OXPHOS [4]. AOX and DHex activation mitigates changes in cellular redox conditions which, if left unchecked, can lead to oxidative stress and damage to cellular components, including nucleic acids [5–7]. Such changes in redox conditions occur, for example, when the cytochrome pathway (CIII–CIV) is impeded by endogenous or exogenous stressors. Accordingly, DHex and AOX have been hypothesized to play general roles in cellular stress responses, especially in plants [4,8–12].

While mt mutation rates are elevated compared to nuclear DNA in most bilaterian animals [13] this trend is reversed in most angiosperm lineages [14]. However, closely related species within the angiosperm genus *Silene* have experienced a relatively recent, rapid increase in mt evolution and have dichotomous mt mutation rates, despite overall similar morphology and ecology [15]. 'Fast' species have mt mutation rates on par with mammals (i.e., mt-DNA evolves faster than N-

48 DNA), while ‘slow’ species show rates similar to typical angiosperms (i.e., mt-DNA evolves
49 slower than N-DNA). The accumulation of slightly deleterious mt mutations in particular is
50 predicted to disrupt mitochondrial function, owing to the uniparental inheritance of mt-DNA and
51 resulting Hill-Robertson like effects [16,17]. Yet, how the recent acceleration in mt mutation
52 accumulation in *Silene* has affected mt physiology is unknown.

53 To illustrate the evolutionary rate differences in *Silene*, in the mt-encoded gene *COX1,* 32 amino
54 acid substitutions have accumulated in the fast species *S. conica* since it shared a common
55 ancestor with *Arabidopsis*, while only seven have accumulated in the slow species *S. latifolia*
56 [15]. Previous work suggests that these substitutions are driven by increased mutation rates and
57 not demographic processes such as a bottleneck in population size– although more definitive
58 tests are needed. Fast species show increases in both nonsynonymous (d_N) and synonymous (d_S)
59 substitutions in mt-encoded genes, but not an elevated d_N / d_S ratio (a hallmark of relaxed
60 selection) [18,19]. Similarly, only N-mt genes show increased d_N / d_S in fast species, whereas all
61 N genes are expected to show increased d_N / d_S after a genetic bottleneck [18,19].

62 In this study we assessed the functional consequences of mutation accumulation on
63 mitochondrial respiration in fast and slow *Silene* species. We calculated flux control factors
64 (FCFs), which describe the capacity of an ETS complex to contribute to mitochondrial
65 respiration [see electronic supplementary material, 25]. If rapid increases in mt mutation rates
66 cause deleterious effects, we predicted that chimeric ETS complexes from fast species would
67 show a lower contribution to respiration than those from slow species. Additionally, we
68 predicted that fast species would show increased reliance on strictly N encoded accessory
69 complexes associated with stress responses.

70 **2. Materials and methods**

71 **(a) Plant care and representative species**

72 We grew 100 plants representing seven 'fast' and six 'slow' *Silene* species in an environmental
73 chamber under fixed light, humidity, and watering schedules to minimize variation from
74 environmental effects, similar to [21]. Multiple accessions were used for many species (i.e.,
75 species were represented by multiple collections when possible). See electronic supplemental
76 material, table S1 for sample sizes, species, accessions, and origins.

77 **(b) Mitochondrial isolation and respirometry**

78 To account for slight variation in growth rates among individuals, we developmentally matched
79 our samples by harvesting plants with mature leaves prior to flowering and included
80 representatives from both speeds on each sampling day. We sampled 1 g of cauline/rosette leaves
81 from each plant for mitochondrial isolation, following [21]. Briefly, leaves were minced, ground
82 in ice cold mt isolation buffer [22] and intact mitochondria were isolated using differential
83 centrifugation.

84 To quantify mt respiration we used an established protocol [21] to measure seven specific
85 aspects of respiratory control of the ETS and overall OXPHOS function using high-resolution
86 respirometry (see electronic supplemental material for details). Briefly, we measured the rate of
87 O_2 consumption from isolated mitochondria from each sample in the presence of specific ETS
88 electron-donors and inhibitors using the Oroboros O2K system (Innsbruck, Austria). From these
89 consumption rates we calculated six flux control factors (FCFs): chimeric core ETS complexes
90 CI and CIV, the entirely N-encoded core ETC complex CII, the N-encoded alternative
91 complexes DHex and AOX, and overall OXPHOS efficiency. OXPHOS efficiency in our
92 protocol is similar to the widely used respiratory control ratio and is calculated as the ratio of

93 respiration rate before and after the addition of ADP [20]. We also recorded the maximum
94 respiration rate observed prior to the addition of ETS inhibitors.

95 **(c) Statistical analyses**

96 We used linear mixed effects models (LMMs) to compare differences in FCFs between fast and
97 slow mutation rates and among species. To control for multiple observations within a species, we
98 included accession as a random factor (see electronic supplemental material for details). We
99 found heteroscedasticity in AOX FCFs between fast and slow rates, so we estimated standard
100 errors separately for each group. We log-transformed the CIV FCF and maximum respiration to
101 meet the assumption of normality. Because FCFs have no meaningful units, we present the
102 results as % change from the slow taxa with 95% confidence intervals (95% CI). We also
103 modeled correlations among FCFs and whether correlations differed in slow vs. fast species by
104 fitting LMMs with the same random factor as above. We performed these analyses and
105 visualization in R 4.0.0. [23]

106 **3. Results**

107 For the entirely N-encoded accessory ETS complexes AOX and DHex, we found that average
108 FCF values for fast *Silene* species were 48.3 % (± 30.9, 95% CI) and 29.1% (± 20.3) greater than
109 slow species, respectively (figure 1b. AOX; $d.f.$ = 71, p = 0.019. DHex: $d.f.$ = 70, p = 0.038).
110 However, FCF values of CII, which is also strictly nuclear encoded but considered a part of the
111 core ETS, were slightly lower in fast species, although not statistically different (figure 1b, table
112 S2, $d.f.$ = 72, p = 0.35). OXPHOS efficiency of fast species was 15.1% (± 7.4) lower than slow
113 species (figure 1b. $d.f.$ = 71, p = 0.005). We found that chimeric ETS complexes, CI and CIV,

and maximum respiration capacity tended to be slightly lower in fast species, although not statistically different (figure 1b, table S2. $p > 0.38$ for all comparisons).

The magnitude of some FCF values were variable among fast species (figure 1b, table S3, S4) such that the overall trends observed between fast vs. slow species are not uniformly distributed across the sampling of species in this study. The greatest differences in FCFs among fast species were in AOX ($p = 0.001$), CII ($p = 0.01$), CIV ($p = 0.02$), and Max respiration ($p = 0.002$) (table S4). *Silene subconica*, and to a lesser extent, *S. grisebachii*, and *S. conica* typically showed greater differences from the slow average than the other fast species (figure 1b).

Among the seven respiratory measures calculated, we found that several were correlated, and that the magnitude of that correlation was similar between fast and slow species in most cases (figure 2a-f, figure S1). However, the strength of the relationship between AOX and DHex depended on mt mutation rate (interaction $p < 0.001$). OXPHOS efficiency was lower when AOX flux was higher in both fast and slow species ($p = 0.002$, interaction $p = 0.44$). CII values increased with DHex in both groups ($p < 0.0001$; interaction $p = 0.72$). Higher CIV, AOX, and DHex FCFs were associated with lower maximum respiration rates in both groups (figure 2d-f; $p < 0.01$ for all; interaction $p > 0.37$ for all).

Discussion

Here we found subtle, yet measurable differences in mt physiology between closely related species with dramatically different mt mutation rates. Overall, mitochondria from fast species performed similarly to slow species, but showed a higher capacity of nuclear-encoded accessory respiratory proteins to contribute to mitochondrial respiration (figure 1b). The greatest difference we found was in AOX capacity, which functions primarily as a stress mediator, preventing mt damage from excessive reactive oxygen species (ROS) production [24–26]. Mitochondria from fast species are possibly primed to mitigate oxidative or other stressors that could impede chimeric ETS function. Electron flow through AOX is typically activated when metabolic flux through the chimeric cytochrome pathway complexes is inhibited [4]. Impeded electron flow through the cytochrome pathway causes an overly reduced ETS and has the potential to produce excessive ROS [27]. A historic, relatively rapid increase in mt mutation rate during *Silene* evolution [15,19] may have caused a dramatic shift in oxidation state due to inhibition of electron flow through the cytochrome pathway [28] resulting in an increased reliance on AOX respiration that is maintained in some contemporary lineages.

Rescue of mitochondrial function from deleterious mt mutations could arise from compensatory changes in N-DNA (e.g., 'mitonuclear coevolution' [29,30]) or plasticity in alternative metabolic pathways. Here we show that nuclear responses to accelerated mt mutation rates in *Silene* may extend beyond molecular evolution, to mt physiology as well. Although there is currently no evidence of positive selection on AOX or accessory NADHs in fast species, the responses observed here may be due to molecular evolution in the nuclear genes encoding these proteins and/or ancestral plastic responses in mt physiology. We speculate that increased AOX respiration allows for adequate ATP production and maintenance of redox homeostasis to prevent the over-

156 production of ROS, in conjunction with complementary N evolution that may act to mitigate
157 harmful effects on chimeric ETS complex function.

158 In addition to the physiological differences in some fast *vs*. slow species we found here,
159 responses to increased mt mutation rates may also include complementary N mutations. Nuclear
160 coevolution in response to elevated mt evolution rates has been well documented in *Silene*
161 [18,19,31]. Fast species show elevated rates of evolution and signatures of positive selection in N
162 encoded, mt-interacting genes [19]. However, co-evolutionary responses in AOX, DHex, and
163 other accessory nuclear mt proteins should be investigated further to complement the changes in
164 mitochondrial physiology observed here.

165 Differences in FCF values attributed to fast mutation rates were subtle and not universal and may
166 be driven by select species (figure 1b). Most notably, *S. subconica* relied heavily on AOX and
167 DHex, supporting our previous comparisons of *S. subconica* and *S. conica* [2]. It is unclear
168 whether mt mutation rates remain elevated in fast species, which may explain interspecific
169 variation if individual species or lineages have slowed to different degrees. Furthermore, low
170 effective population size in some fast species like *S. subconica* may cause inefficient selection on
171 mt genes and further reliance on nuclear-encoded complexes. Future work could focus on
172 examining species-level differences in mitochondrial dynamics, including quantifying ETS
173 protein content and gene expression, within fast species.

174 We found that higher AOX values were associated with lower OXPHOS efficiency across
175 mutation speeds (figure 2a). Electron flow through AOX is inherently inefficient for OXPHOS
176 because AOX does not translocate protons across the inner mitochondrial membrane required for
177 phosphorylation of ADP [8]. Additionally, flux through AOX bypasses CIII-CIV, which do
178 translocate protons, leaving only CI to contribute to the requisite proton gradient (figure 1a). We

also found that the correlation between AOX and DHex flux was stronger in fast species than slow species (figure 2b). DHex and AOX can form a supercomplex which cycles electrons rapidly to shift the redox balance to an oxidized state, which disfavors ROS production [26]. Therefore, it is possible that increased reliance on AOX and DHex in fast species is a plastic nuclear response to oxidative stress caused by mt mutation accumulation. This agrees with previous studies implicating these alternative ETS complexes in environmental stress responses [9,25,32,33] but extends the response to "domestic" stressors.

Predictions about the impact of mt mutations on mt function are varied: efficient mt selection results in fixation of only neutral mutations and has recently been suggested to be common despite classic mutation accumulation theory [34–36]. Alternatively, compensatory evolution in the nuclear genome may offset deleterious mt mutations, making them effectively neutral (or even advantageous) [37,38]. Here, we find evidence that despite drastically elevated mt mutation rates, overall mt function is minimally impacted in fast *Silene* species. This is likely due to both mitonuclear coevolution and nuclear-mediated responses in mt physiology, most notably increased reliance on AOX. Our results highlight the importance of considering physiological outcomes when making predictions about the importance of mitonuclear interactions.

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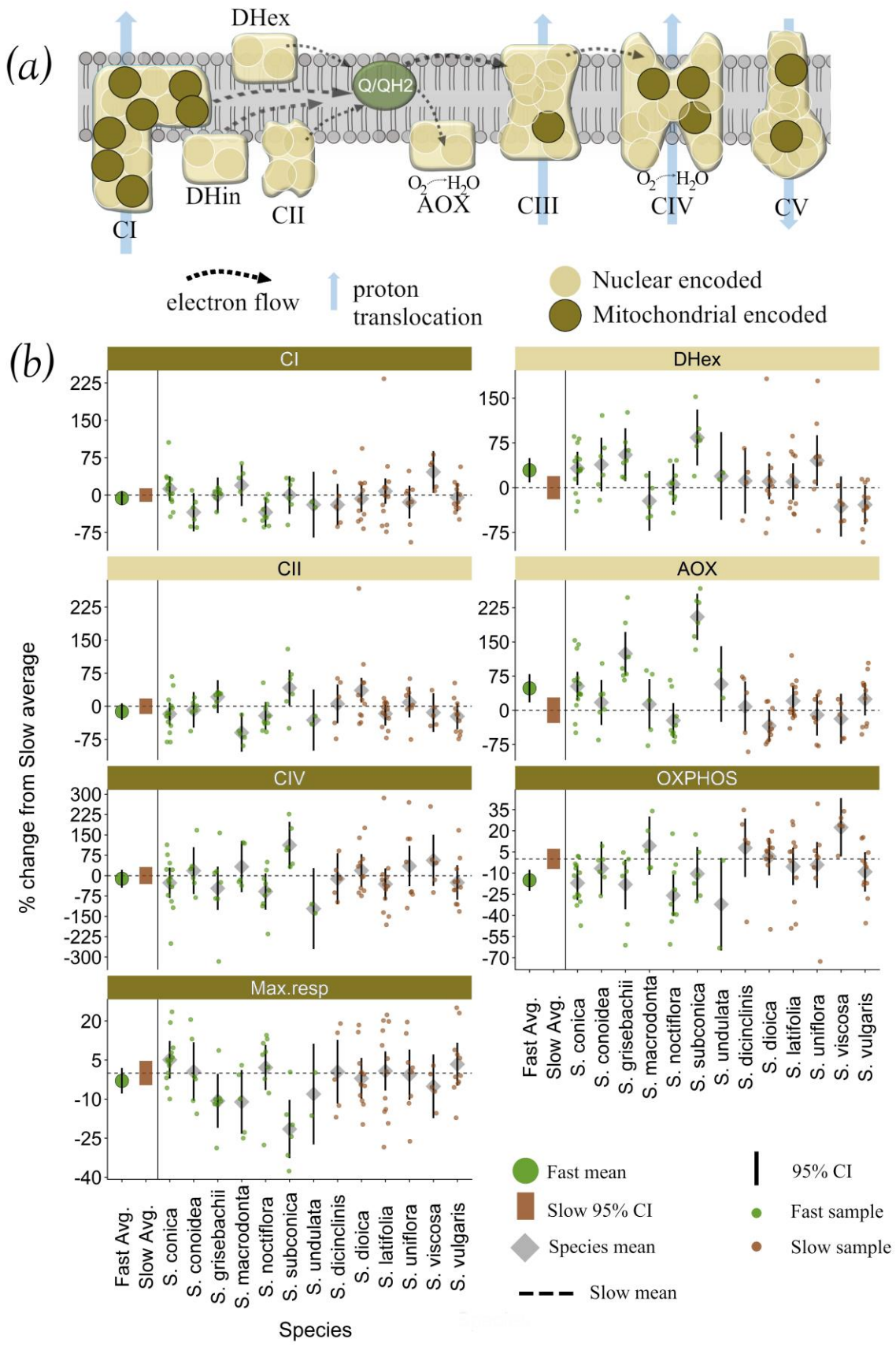
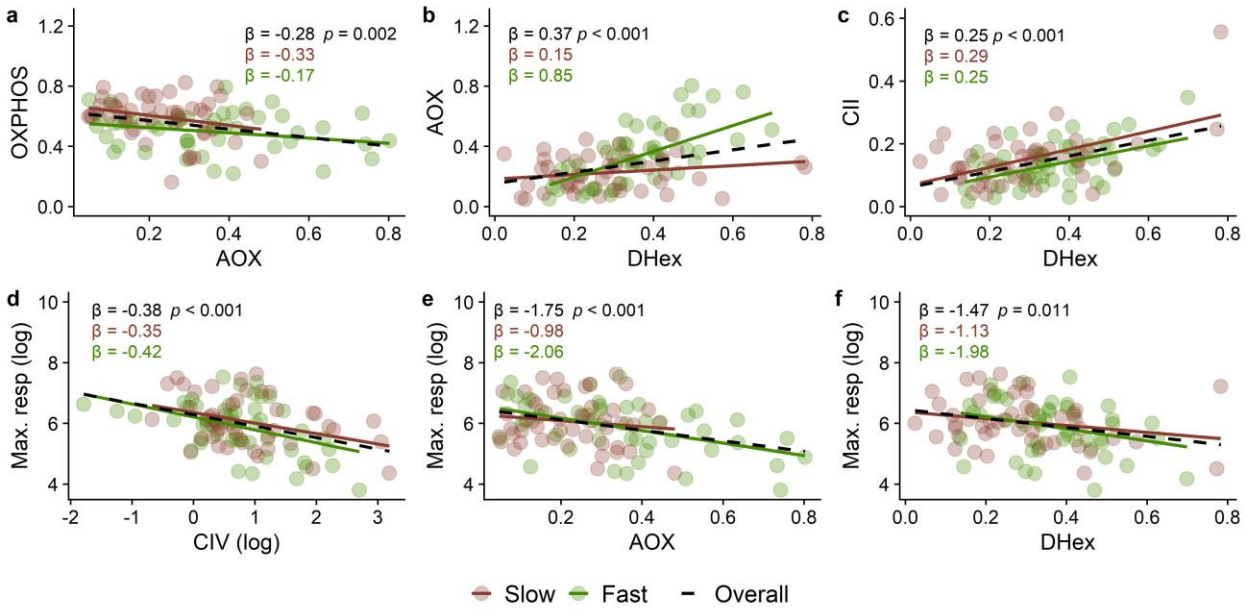
Figure Captions

Figure 1. (a) Overview of relevant aspects of plant mitochondrial electron transport chain components. Electrons enter through complex I, (CI), CII, or the alternative NADH dehydrogenases (DHex/in) and follow either the alternative oxidase pathway (AOX) or the cytochrome pathway CIII – CIV to reduce oxygen to water. CI, CIII, and CIV translocate protons to establish the protonmotive force used by CV for ATP production. (b) Differences in mitochondrial (mt) flux control factors (FCF) and maximum respiration between *Silene* species with slow or fast evolving mt-DNA. Means with 95% confidence intervals (CI) that do not overlap the slow mean are statistically different at $\alpha = 0.05$.

Figure 2. Relationships among FCF types in *Silene* with different mitochondrial mutation rates. Dashed lines show the relationship between the two variables overall from linear mixed effects models. The brown circles and line show the individual samples and model estimated slope for the slow species and the green circles and line show the same for the fast species.


(a)
DHex
Q/QH2
DHin
CI
CII
AOX
CIII
CIV
CV
electron flow
proton translocation
Nuclear encoded
Mitochondrial encoded
(b)
CI
DHex
CII
AOX
CIV
OXPHOS
Max.resp
% change from Slow average
Fast Avg.
Slow Avg.
S. conica
S. conoidea
S. grisebachii
S. macrodonta
S. noctiflora
S. subconica
S. undulata
S. dicinclinis
S. dioica
S. latifolia
S. uniflora
S. viscosa
S. vulgaris
Species
Fast mean
Slow 95% CI
Species mean
Slow mean
95% CI
Fast sample
Slow sample

328


a
β = -0.28 p = 0.002
β = -0.33
β = -0.17
OXPHOS
AOX
b
β = 0.37 p < 0.001
β = 0.15
β = 0.85
AOX
DHex
c
β = 0.25 p < 0.001
β = 0.29
β = 0.25
CII
DHex
d
β = -0.38 p < 0.001
β = -0.35
β = -0.42
Max. resp (log)
CIV (log)
e
β = -1.75 p < 0.001
β = -0.98
β = -2.06
Max. resp (log)
AOX
f
β = -1.47 p = 0.011
β = -1.13
β = -1.98
Max. resp (log)
DHex
Slow
Fast
Overall

329